FACTORS CONCERNED IN THE REGULATION OF THE DEHYDROASCORBIC ACID CONTENT OF HUMAN BLOOD

A thesis presented for the Degree of Doctor of Philosophy

by

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

HISTORICAL BACKGROUND.

The first description of scurvy is accredited to Hippocrates who reported symptoms of the disease in soldiers. It ravaged the ranks of the armies of the Crusaders, but it was as a mariners' disease that it was most prominent in the medieval world.

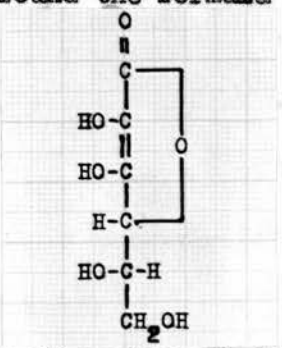
The efficacy of watercress, Icelandic scurvy grass and citrus fruits in curing symptoms had been recognised several centuries ago, but the interpretation of the function of these vegetables and fruits was not achieved until comparatively recently. At the beginning of the twentieth century it was realised that scurvy was one of a group of "deficiency diseases" that could be cured by the feeding of small amounts of a "vitamin" or "accessory food factor." The appropriate vitamin was termed "vitamin C" and recognised to be found in quantity in citrus fruit juices.

The vitamin was isolated from lemon juice and its chemical nature established in the years 1930-33. It was found to be a strongly reducing hexuronic acid, subsequently being termed "ascorbic acid" by agreement between the principal workers in the field. Synthesis of the biologically active ascorbic acid from xylosone, itself completely inactive as an antiscorbutic, finally established conclusively that ascorbic acid was the antiscorbutic dietary factor (Chick, /

(Chick, 1953; Hirst, 1953; King, 1953).

OXIDATION OF ASCORBIC ACID TO DEHYDROASCORBIC ACID.

The structural formula of ascorbic acid was established by several workers, chief amongst them being the Birmingham School in this country (Herbert, Hirst, Percival, Reynolds and Smith, 1933) and Karrer (1933) and Micheel (1933) on the Continent. Their experimental material was ascorbic acid prepared from adrenal glands and paprika supplied largely by Szent-Györgyi. They found the formula to be as shown below:

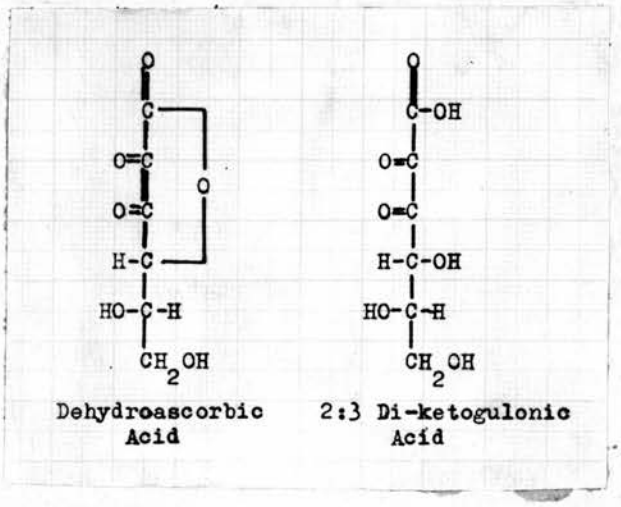


L-ascorbic acid is the antiscorbutic form.

The chemical feature of ascorbic acid which is of most interest to the biochemist is its susceptibility to oxidation in which molecular oxygen is the final hydrogen acceptor. Though the reaction has been widely studied, knowledge of the course of the oxidation, which culminates in the formation of water and carbon dioxide, is scanty and incomplete. Haworth's colleagues in 1933 were the first investigators to produce an adequate account of the steps involved (Herbert et al., 1933). They showed the primary oxidation product to be dehydro-

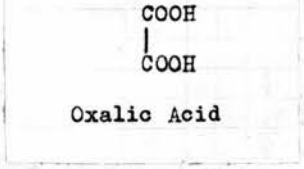
ascorbic /

ascorbic acid, which can be readily reduced to ascorbic acid again by such agents as hydrogen sulphide (Tillmans, Hirsch and Dick, 1932a; Johnson, 1933) and hydriodic acid. Dehydroascorbic acid is very labile in solution and at pH values greater than 4 is converted irreversibly in a non--oxidative reaction to 2:3 di-ketogulonic acid. Subsequent stages in the conversion to water and carbon dioxide have not been fully worked out but some are known to be non-oxidative in character. Oxalic acid and threonic acid are intermediate products in the course of the reaction. No other intermediate has been isolated or identified.

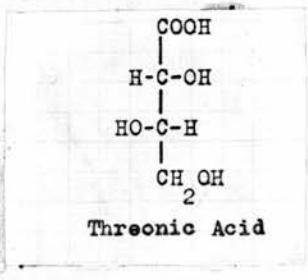


Dehydroascorbic Acid

2:3 Di-ketogulonic Acid



Oxalic Acid



Threonic Acid

The oxidation of ascorbic acid is influenced by hydrogen ion concentration, the rate increasing with increase in pH. The rate also increases with an increase in temperature. The oxidation is light-sensitive as shown by Martini and Malatesta (1946).

PROPERTIES. /

PROPERTIES OF DEHYDROASCORBIC ACID.

The chemistry of dehydroascorbic acid has been little studied, due partly to its lability and the difficulty of estimation, and partly to the fact that it appeared to be of secondary importance compared with the more stable reduced ascorbic acid. (When referring to ascorbic acid in contradistinction to dehydroascorbic acid, it is customary to use the term "reduced ascorbic acid" to prevent confusion.) Moll and Wieters (1936) reviewed the chemistry of this first isolated oxidation product and also gave a method for its preparation in aqueous solution by oxidation of ascorbic acid with benzoquinone. Preparation of the solid substance was not accomplished until 1948 when Kenyon and Munro (1948) prepared it from ascorbic acid by iodine oxidation. They also reviewed some of its physical characteristics. Pecherer (1951) published a modified version of their method, using chlorine as the hydrogen acceptor.

When ascorbic acid solution is submitted to mild oxidation, the dehydroascorbic acid so produced can be reduced quantitatively to ascorbic acid again by hydrogen sulphide treatment, if this is carried out immediately. If the solution of dehydroascorbic acid is allowed to stand before H_2S reduction is carried out, the recovery of the original ascorbic acid is less than quantitative, showing decomposition of dehydroascorbic acid has occurred as the solution stands. /

stands. The decomposition is progressive with time and for good recovery of the original ascorbic acid it is necessary to treat with hydrogen sulphide in as short as possible time after formation of the solution of dehydroascorbic acid.

Moll and Wieters (1936) also investigated the decomposition of dehydroascorbic acid and showed it to be non-oxidative, taking place as rapidly in an anaerobic as in an aerobic atmosphere. They showed that the decomposition of dehydroascorbic acid was affected by temperature, the rate of decomposition increasing with increase in temperature. If stored at -4° C, a 6% solution of dehydroascorbic acid decomposed only slowly; 95% of the theoretical concentration of ascorbic acid was recovered after H_2S reduction, 5 days after its preparation. No matter how far the temperature was lowered, however, these authors reported that complete stability of dehydroascorbic acid solution was never achieved.

As decomposition of dehydroascorbic acid proceeds there appears at the same time a substance which, like reduced ascorbic acid, reduces dichlorphenolindophenol, silver nitrate and iodine readily. It is not, however, antiscorbutic, and so is not the original reduced form of the vitamin. There is never more of the substance formed than the equivalent of 10% of the original dehydroascorbic acid. Moll and Wieters have suggested that it is one of the reductones, a class of substances which has similar reducing properties to ascorbic acid.

The /

The main characteristics by which dehydroascorbic acid is identified are:

1) Its ready reduction to ascorbic acid, and

2) The formation of a phenylhydrazone on dinitrophenylhydrazine treatment.

Reaction 2) was first observed by Herbert and his colleagues (Herbert et al., 1933) and by Ohle (1934). The hydrazone is reported to be formed only after the conversion of dehydroascorbic acid to diketogulonic acid, but the conditions of the reaction favour this hydration (Penney and Zilva, 1943).

According to Moll and Wieters (1936), dehydroascorbic acid also reacts very smoothly with various phenylenediamines to give solid yellow condensation products.

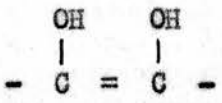
FACTORS AFFECTING THE OXIDATION OF ASCORBIC ACID.

Since ascorbic acid was readily oxidisable and was known to be very generally distributed in the animal body and in plants, it seemed probable that this compound might be concerned in the maintenance of oxidation-reduction equilibrium in cellular reactions. This postulate prompted various workers to study the kinetics of the oxidation in aqueous solution, but at first with indifferent success. It was a sluggish system in which to carry out potential determinations and the instability of dehydroascorbic acid hindered determinations further. There was no satisfactory establishment /

establishment of the thermodynamic reversibility of the oxidation of ascorbic acid to dehydroascorbic acid until the work of Barron, De Meio and Klemperer (1936b). These investigators concluded that, in pure solutions, ascorbic acid was only autoxidisable in alkaline media, the autoxidation increasing with increase in pH. Borsook and his co-workers (Borsook, Davenport, Jeffreys and Warner, 1937) succeeded in establishing the potentials for the oxidation and showed conclusively that AA = DHA + $2H^+$ was the only step in the whole oxidation chain that was physiologically reversible.

Barron et al. (1936b) were also the first to estimate quantitatively the great sensitivity of the system to metallic ions. They reported that there was a catalytic action in a solution containing a concentration of cupric ions as low as 46µg./litre. The system was also found to be very sensitive to ferric ion catalysis. Since both these metallic ions are found widespread biologically in the free state and protein-bound, there has been much interest in their effect on the kinetics of the oxidation. Most work reported in the literature is on copper catalysis. The conclusion is that copper enters the reaction as the cupric salt, is reduced to the cuprous form with accompanying formation of dehydroascorbic acid and is eventually regenerated in the original cupric form, hydrogen peroxide also being /

being produced (Dekker and Dickinson, 1940; Silverblatt, Robinson and King, 1943). The copper catalysis is very sensitive to pH, and according to Weissberger and LuValle (1944), the oxidation of the monovalent ion of ascorbic acid is the one which is sensitive to the catalytic action of copper. The presence of the dienol grouping



appears to be necessary for effective copper catalysis.

Numerous anions affect the sensitivity of ascorbic acid solution to oxidation, some by acting directly on the vitamin and others indirectly through their action on cupric and ferric ions. Trichloracetate was early recognised as a stabilising anion, as was the more efficient metaphosphate. The inhibitory action of the latter acid on copper catalysis of the oxidation is exploited in the usual method for estimating the reduced form of ascorbic acid. Fujita and Iwatake (1935) introduced the use of metaphosphate and it was further examined by Musulin and King (1936) and by Lyman, Schultze and King (1937). Lyman and his co-workers showed that its inhibitory action depends upon a depression of the copper ion concentration, and in addition, upon a lowering of the pH of the solution. They reported that in buffered solutions near pH 7, there is no appreciable inhibition of ascorbic acid oxidation by the addition of metaphosphate. /

phosphate. They also demonstrated the accelerating action of chloride on the oxidation, a fact also pointed out by Moll and Wieters (1936). Mapson (1941, 1945) extended this work and showed that the halides as a class increase the rate of ascorbic acid oxidation, iodide being the most effective; fluoride, however, is inactive. The anion increases the rate of the following reaction in the catalytic oxidation:

$$2Cu^{++} + AA = 2Cu^{+} + DHA + 2H^{+}$$

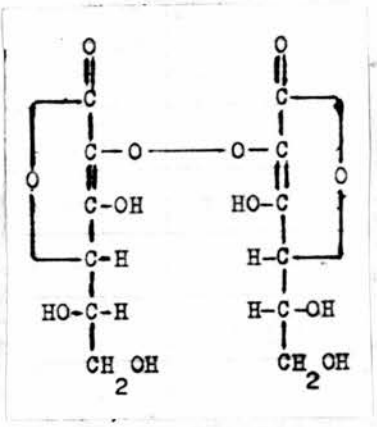
O-iodobenzoic acid has also been reported to increase the oxidation rate, the effectiveness varying with the buffer used (Hellerman and Caraway, 1953).

Various buffers have been examined for their action on the oxidation. In general, it is believed that buffer solutions stabilise ascorbic acid solutions to a certain degree (Moll and Wieters, 1936). It has been claimed that the rate of oxidation of ascorbic acid at pH 7 is less in the presence of citrate than in the presence of phosphate (Sukhenko, Priss and Radushkevich, 1951). Rosenfeld (1943) has suggested that in the presence of phosphate, at pH 7, dehydroascorbic acid is not converted to diketogulonic acid, but that there is a scission of the 6-carbon chain with a quantitative formation of oxalic acid. Finally, De Caro and Giani (1934) have mentioned the stabilising action of Ringer's solution on the system.

THE /

THE EXISTENCE OF MONODEHYDROASCORBIC ACID.

Bezssonoff and Woloszyn (1936) employed Martini and Bonsignore's (1934) method for the estimation of ascorbic acid, using methylene blue. They found in the course of their work that the oxidation of ascorbic acid by the dye took place in two stages, and on these grounds suggested the existence of an intermediate which they called monodehydro-ascorbic acid. The structure of the intermediate they suggested to be as follows:

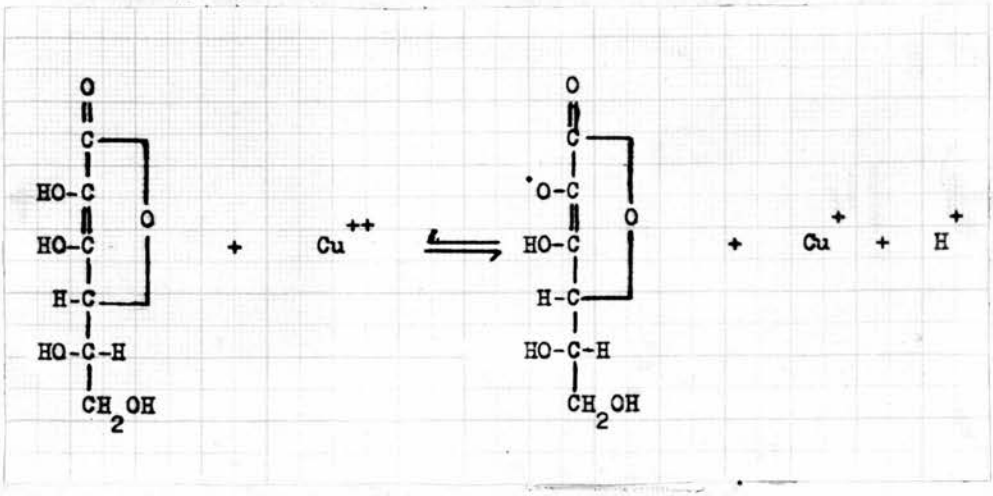


(Bezssonoff and Woloszyn, 1936)

In 1939, these authors (Bezssonoff and Woloszyn, 1939) proposed a method for the estimation of monodehydroascorbic acid in tissues, using both indophenol and methylene blue estimations. They claimed that indophenol was reduced by ascorbic acid and by monodehydroascorhic acid, while methylene blue reduction was due only to reduced ascorbic acid. The semi-oxidised form could therefore be estimated by subtracting /

subtracting the methylene blue value from the indophenol value.

Dekker and Dickinson (1940) and Silverblatt et al. (1943) reported that the copper catalysed oxidation of ascorbic acid by molecular oxygen was a two-stage reaction. Weissberger and LuValle (1944) suggested that the intermediate in this oxidation was a free radical. Two of these could form a semiquinone of the type conceived by Bezssonoff and Woloszyn (1936).



Such a free radical would accept one electron very rapidly to form the reduced ascorbic acid ion, or would equally rapidly form dehydroascorbic acid with the loss of another hydrogen atom and rearrangement of the electron field.

The oxidation of tyrosine was found to be impaired in scorbutic guinea-pigs (Sealock and Silberstein, 1940), in premature babies (Levine, Gordon and Marples, 1941) and in scorbutic /

scorbutic adults on a high tyrosine diet (Rogers and Gardner, 1949). The defect was shown by a high urinary excretion of "tyrosyl" compounds, which was decreased on administration of ascorbic acid.

Neuberger (1949) suggested that tyrosine was attacked in normal biological oxidation by a cationoid reagent at the ortho- and para- positions relative to the hydroxyl group. This stage seemed to be the one sensitive to the lack of ascorbic acid. Lloyd and Sinclair (1953) extended this hypothesis with their suggestion that the responsible cationoid reagent might be the free radical monodehydro-ascorbic acid.

These latter authors also postulated that vitamin C acted in the form of the free radical in relation to the metabolism of the adrenal cortex, and to the secretion of the adrenal cortical hormone.

The existence of such a free radical would explain biological reactions of vitamin C otherwise difficult to interpret. However, Lloyd and Sinclair (1953) stressed the need for further experimental work before accepting this hypothesis of a form of ascorbic acid which acted as an oxidising agent.

BIOLOGICAL /

BIOLOGICAL OXIDATION OF ASCORBIC ACID.

Since so much work has shown the susceptibility of ascorbic acid in aqueous solution to oxidation, it is obviously of the greatest importance to estimate the effect of biological media on its stability.

Ascorbic acid was isolated from biological material in the reduced form. When solutions of the vitamin were incubated with various tissue extracts or biological fluids, the vitamin was stable far longer than in pure solution (Quastel and Wheatley, 1934; Kellie and Zilva, 1935; Mawson, 1935). Green (1933) postulated the existence of a stabilising mechanism within the body which was responsible for this fact. De Caro and Giani (1934) pursued this idea and suggested the involvement of glutathione. Mawson (1935) presented the theory that cysteine and cystine were also concerned in the stabilisation but he felt this was not the entire explanation. According to Kellie and Zilva (1935) an explanation of the stabilising action of tissue extracts could be sought in their inhibition of copper and iron catalysis.

Mawson's (1935) conclusions, as they stood, were of little significance, since they were based on work done under non-physiological conditions. In 1936, Barron and his colleagues (Barron, Barron and Klemperer, 1936a) realised this and so re-investigated the possibility that glutathione was /

was a biological stabilising agent of ascorbic acid. They studied various fluids - cerebrospinal fluid, vitreous humour, gastric juice, urine, serum and whole blood - and concluded that in them stabilisation of ascorbic acid occurred because of an inhibition of copper and iron, and also that glutathione helped in this way to stabilise the vitamin by forming an inactive unionised copper-glutathione complex. They reported the same degree of protection of ascorbic acid in blood serum and in whole blood and so concluded that the cellular portion of blood had no especial protective capacity. This was opposite to the conclusions of Kellie and Zilva (1935) who stated that leucocytes were the only blood components inactive in the stabilisation of ascorbic acid. Borsook et al. (1937) also disagreed with Barron's results (Barron et al., 1936a) in their finding of the greater protection from whole blood than from serum. Later work demonstrated that whole unhaemolysed blood was indeed more efficient than serum or plasma in the stabilisation of ascorbic acid (Kassan and Roe, 1940; Heinemann, 1941; Golden and Garfinkel, 1942). Borsook and his co-workers (Borsook et al., 1937) also reported that glutathione could stabilise ascorbic acid in solution, but attributed this to a direct reducing action on any dehydroascorbic acid that might form.

While /

While glutathione is regarded generally as the principal biological stabilising factor, various other substances have been considered. Giri and Krishnamurthy (1941) suggested the possible protective action of some purines and of creatinine. Nevertheless, glutathione is the substance most generally accepted as stabilising agent for ascorbic acid in biological fluids and extracts. Indeed, Szent-Györgyi (1928) mentioned that it could reduce his hexuronic acid and, as has been indicated, various authors (De Caro and Giani, 1934; Mawson, 1935; Goldstein and Volkenzon, 1938) regarded it as the principal, while others claimed it to be the only factor in the stabilisation (Borsook et al., 1937).

The fact that the biological distribution of glutathione is very similar to that of ascorbic acid is an additional support to this idea. The work on their interdependence has progressed further in the field of plant metabolism, where several investigators have established a stoichiometric relationship controlled by ascorbic acid oxidase. This enzyme is believed only to occur in plants (Hopkins and Morgan, 1936; Crook and Hopkins, 1938; Crook, 1941).

In animal metabolism, the situation is less clearly defined. In 1935, Bersin and his colleagues (Bersin, Köster and Jusatz, 1935) showed that scurvy depleted the level of ascorbic acid and glutathione in the guinea-pig adrenal, and that restoring the deficient vitamin to the diet raised the level /

level of both in the gland. They found no change in blood glutathione in scurvy. On the other hand, Prunty and Vass claimed a fall in human blood glutathione occurred as the ascorbic acid level rose (Prunty and Vass, 1943). Although not contradictory, these results seem hardly compatible.

Oxidation of ascorbic acid does occur in biological media. Haemochromagens oxidise ascorbic acid very readily (Barron et al., 1936a; Lemberg, Legge and Lockwood, 1939). This occurs only in the presence of copper ions. When whole blood is laked, the resulting release of haemoglobin into a medium containing copper ions is responsible for complete oxidation of all ascorbic acid present in the blood and subsequent errors in estimation.

There is no evidence as yet to suggest a parallel enzyme to ascorbic acid oxidase in animal tissues. In 1937, Stotz and his fellow workers (Stotz, Harrer, Schultze and King, 1937) oxidised ascorbic acid by adding to it a brei prepared from guinea-pig livers. The oxidation was not due to the copper content of the brei. They suggested the reaction mechanism was by means of the indophenol oxidase--cytochrome c enzyme system, since cyanide, carbon monoxide and azide all inhibited the oxidation. Ruffo (1952) reported that a paste formed from the livers of fasting rats accelerated ascorbic acid oxidation in alkaline conditions. However, this work is of little physiological importance.

BIOLOGICAL /

BIOLOGICAL REDUCTION OF DEHYDROASCORBIC ACID.

a) In vitro.

Not only do tissues such as liver, kidney and adrenal stabilise ascorbic acid, but they also reduce dehydroascorbic acid to reduced ascorbic acid. This has been reported by several workers, in particular Borsook and his colleagues (Borsook et al., 1937) and Schultze, Stotz and King (1937). Both stabilisation and reduction are generally recognised to be due to the same reaction mechanism. Although Borsook et al. (1937) reported ascorbic acid to be much more stable in whole blood than in plasma or aqueous solution, they failed to find any reduction capacity in whole blood and similarly, found plasma to be inactive. The inability of plasma to reduce dehydroascorbic acid has been amply confirmed by Plaut and Bülow (1935) and by Schultze et al. (1937) but the latter, unlike Borsook et al. (1937) found that whole blood and erythrocytes' suspensions would reduce added dehydroascorbic acid. This has been confirmed by Lloyd (1951), Lloyd and Parry (1954) and by the present writer.

While most authors regard the reduction of dehydroascorbic acid in animals as due wholly or in part to the action of glutathione, there has been some difference in views as to the reaction mechanism concerned. Roe and Barnum (1936) showed fluoride inhibited the reduction of dehydroascorbic /

dehydroascorbic acid and that plasma coagulated by heat was inactive with respect to the reduction. On this basis, they claimed that the reduction was enzymic. However, Plaut and Bülow (1935) and Schultze et al. (1937) demonstrated that plasma could not reduce dehydroascorbic acid under ordinary conditions. Also fluoride is a non-specific inhibitor. On these grounds, Roe and Barnum's (1936) claim has little experimental support and it remains an open question that the reduction is enzymic. Subsequent workers are agreed on the participation of sulphydryl groups, since specific -SH poisons, iodoacetate and arsenite, are found to inhibit the reduction completely (Borsook et al., 1937; Schultze et al., 1937).

The reaction actually taking place in biological media between dehydroascorbic acid and glutathione does not appear to be the most obvious one expressed in equation (1).

$$DHA + 2GSH = AA + GSSG \quad (1)$$

This is a third order reaction and would take place much more slowly than the results obtained with biological systems suggest actually happens. Schultze and his fellow workers (Schultze et al., 1937) found the relationship between the two substances did not fit equation (1). Mawson (1935) noted that far less glutathione was used in dehydroascorbic acid reduction than was needed on the basis of this equation. Borsook and his colleagues (Borsook et al., 1937) suggested that /

that there was a glutathione-dehydroascorbic acid complex formed but gave no further details of its possible structure or reactivity. Kinkawa (1944) claimed that there was another reducing mechanism in animal tissues, in addition to glutathione and fixed -SH. This unidentified substance he reported to be non-enzymic and heat-stable. It was also claimed to be water-soluble and ether-insoluble.

There are various factors said to influence the extent of the reduction. If the concentration of glutathione in the medium is increased, then the reduction increases. An increase in pH has the same effect (Borsook et al., 1937). Schultze et al. (1937) found that an increase in ascorbic acid concentration caused a fall in the amount of dehydro-ascorbic acid reduced.

There is obviously some mechanism in biological systems, other than the glutathione present, which is responsible for the reduction of dehydroascorbic acid. The problem arises whether such a mechanism acts independently of glutathione or whether its part in the reduction is closely related to the reducing action of glutathione, possibly in the regeneration of reduced glutathione from the oxidised form, and so in the maintenance of the concentration of the tripeptide.

The previous condition of the animal whose tissues are under examination may be important. A decreased reduction

was /

was reported (Vinokurov and Silakova, 1944; Matusis, 1951) in the tissues of people who had been chronically ill prior to death, compared with those whose health had been good. Schroll (1939) reported that tissues from animals trained on a treadmill reduced dehydroascorbic acid more rapidly than those from untrained animals. Parrot and Gazave (1951) claimed that liver from guinea-pigs pre-fed on a catechol--rich diet had an increased reduction capacity.

b) In vivo.

The reversibly oxidised form of vitamin C was recognised to be antiscorbutic in humans and guinea-pigs even before the chemical nature of the vitamin was fully established (Zilva, 1928; Tillmans, Hirsch and Hirsch, 1932b; Demole, 1933; Hirst and Zilva, 1933). Bioassay on guinea-pigs (Fox and Levy, 1936; Moll and Wieters, 1936; Gould and Schwachman, 1943), human excretion studies (Johnson and Zilva, 1934; De Ritter, Cohen and Rubin, 1951) and plasma level and urinary excretion examinations in humans (Todhunter, McMillan and Ehmke, 1950; Clayton, McSwiney and Prunty, 1954) have provided ample support for this.

There is thus corroboration by in vivo experiments for the conclusion based on results from in vitro experiments that a biological mechanism for reducing dehydroascorbic acid does exist. There is, however, a certain amount of controversy about the efficiency of this mehhanism. Roe

and /

and Barnum (1936) state that dehydroascorbic acid is only 25% as active as ascorbic acid, while Borsook et al. (1937) report oxidised orange juice in humans is as efficiently utilised as ordinary orange juice. Gould and Schwachman (1943) find 80% utilisation, weight for weight, of dehydroascorbic acid administered to guinea-pigs, and analysis of the results of Todhunter and her colleagues for humans suggests a similar value (Todhunter et al., 1950).

Analysis of tissue levels after dehydroascorbic acid administration indicates a similar lessening of efficiency with regard to ascorbic acid storage (Bűlow, 1936; Fox and Levy, 1936; Damron, Monier and Roe, 1952). Penney and Zilva (1943) suggest that if small amounts of dehydroascorbic acid are given, there is a quantitative conversion to ascorbic acid and utilisation as the vitamin, but on administration of large amounts there is a decrease in the utilisation. They attribute this to a delay in absorption of dehydroascorbic acid, with consequently greater opportunity for the opening of the lactone ring to form diketogulonic acid. This substance is realised to have no antiscorbutic activity at all (Hirst and Zilva, 1933; Borsook et al., 1937; Penney and Zilva, 1943). The presence of considerable amounts of di-ketogulonic acid in Roe and Barnum's (1936) "dehydroascorbic acid" preparation would explain the low figure they quote for its biological efficiency.

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On the basis of this work, it seems improbable that dehydroascorbic acid is absorbed unchanged, even in part. However, Linksweiler (1954) reports the presence of 0.2mg./100ml. dehydroascorbic acid in blood after its administration to humans, as against 0.08mg./100ml. after a parallel dosage of ascorbic acid in comparable time. It is doubtful if these results are entirely reliable since there are no details of the method of estimation. The author's report of significant excretion of dehydroascorbic acid after its ingestion is in conflict with the work of Johnson and Zilva (1934) and De Ritter et al. (1951) who state there is no appreciable amount of urinary dehydroascorbic acid in similar circumstances.

It can safely be stated that by far the greater part of dehydroascorbic acid, administered to either guinea-pigs or humans, is reduced in the body and utilised as ascorbic acid.

EXISTENCE OF ASCORBIC ACID AND DEHYDROASCORBIC ACID IN BLOOD.

The discovery of the chemical nature of the scorbutic vitamin promoted the search for it in biological material. It had been isolated both from plant and animal material prior to the elucidation of its chemical identity, and this prompted investigation of its presence in blood and urine. The primary investigation technique utilised dichlorphenol-indophenol which had proved a satisfactory rule-of-thumb agent /

agent in the isolation work on lemon juice (Tillmans, 1930). The reduction of the dye was, however, by no means specific for ascorbic acid, and so it was realised that conclusions based on this test alone were insufficient. Van Eekelen, Emmerie, Josephy and Wolff (1933) showed that filtrates prepared with trichloracetic acid from guinea-pig blood, and reduced with hydrogen sulphide, could reduce indophenol, after excess hydrogen sulphide was removed. This capacity was lost if the guinea-pigs became scorbutic, and was increased if the guinea-pigs had previously been on a high ascorbic acid diet. When the filtrate was treated with lead acetate in alkaline solution, a substance was precipitated which dissolved in acid to give a solution reducing indophenol. Since ascorbic acid behaved thus, van Eekelen et al. (1933) concluded this substance in the blood filtrate was the vitamin. Similarly, a substance was precipitated and examined in urine and their conclusion that this, too, was ascorbic acid was substantiated by its isolation from urine as the dinitrophenylhydrazone by Drumm, Scarborough and Stewart (1937).

There has been no serious quarrel with the concept that ascorbic acid is present in blood, but concerning dehydro-ascorbic acid, opinions are divergent. Until a completely satisfactory method is devised for the specific estimation of ascorbic acid, distinguishing it from its oxidation products, /

products, it seems the controversy about the presence of dehydroascorbic acid will continue.

Some early workers believed the vitamin was found in its entirety in the dehydro-form in blood (van Eekelen et al., 1933; Plaut and Bülow, 1935; Fujita and Ebihara, 1937), while others stated that there was no dehydroascorbic acid in blood at all (Kellie and Zilva, 1936; Borsook et al., 1937; Lund and Elmby, 1938). More recently, it has been recognised that it is impossible to be categorical about its presence or absence while methods of estimation are still relatively unspecific. The concensus of recent opinion seems to be that a little of the dehydro-form of the vitamin may be found in the blood, with an increase in the amount in various pathological conditions (Lloyd, Sinclair and Webster, 1945; Chen and Schuck, 1950; Davey, Wu and Storvick, 1952; Banerjee and Belavady, 1953; Stewart, Horn and Robson, 1953; Linksweiler, 1954). While it is rash to state definitely the actual amount, it seems highly improbable that in plasma, it is ever much in excess of 0.20-0.25mg./100ml. and more usually it is in the range 0.05-0.15mg./100ml.

Analysis of the white blood cell-platelet fraction of blood - Crandon's "buffy layer" - has not been concerned with the possible presence of dehydroascorbic acid and the methods used for the estimation of the vitamin in this fraction do not permit a differentiation between the reduced and /

and reversibly oxidised forms of the vitamin. Kellie and Zilva (1935) reported that this portion of the blood could not stabilise ascorbic acid, and Lloyd (1951) suggested that leucocytes could oxidise the vitamin. This suggested that in this portion of blood, the vitamin was found to a large extent, if not wholly, as the dehydro-form.

Relatively few analyses have been carried out for ascorbic acid or dehydroascorbic acid on the erythrocyte portion of blood and here again there appears to have been no attempt to estimate the dehydro-form itself. Technical difficulties are possibly responsible for this omission.

It is improbable that much of the vitamin in the erythrocytes is present in the reversibly oxidised form. With methods currently in use, estimations on whole blood, with subsequent corrections for plasma levels of the two forms of the vitamin, are not sufficiently reliable to permit confirmation of the presence or absence of the dehydro-form in erythrocytes. Although an exhaustive study has not been carried out, the results obtained from erythrocyte determinations in the course of the work for this thesis suggest that there is virtually no dehydroascorbic acid in this fraction of human blood.

PASSAGE /

PASSAGE OF ASCORBIC ACID AND DEHYDROASCORBIC ACID ACROSS BLOOD CELL MEMBRANES.

Results of the investigations on the ability of ascorbic acid to pass across the blood cell membrane were at first inconclusive and confused. There was general agreement that such a transfer took place *in vivo* (Heinemann, 1938; Crandon, Lund and Dill, 1940), but the results were not so clear-cut in *in vitro* work. Borsook *et al.* (1937) stated that ascorbic acid added to blood was still to be found in its entirety four hours later in the plasma, while Heinemann and Hald (1940) and also Butler and Cushman (1940) claimed that a slow passage of ascorbic acid took place from the plasma into the cell and that this was enhanced by shaking. Later, Heinemann (1941) qualified his reports and showed that the presence of leucocytes was necessary for this transfer to be effected. The transfer found to occur *in vivo* was, he explained, therefore, since there were always leucocytes present. Lloyd (1951) verified that ascorbic acid was taken up by leucocytes *in vitro* and stated that, within them, it was oxidised to dehydroascorbic acid.

Golden and Sargent (1952) showed that ascorbic acid did not pass into erythrocytes *in vitro*. Dehydroascorbic acid, on the other hand, was observed to pass quickly into the erythrocytes *in vitro* and there to undergo partial reduction (Panteleeva, 1950; Lloyd, 1951; Lloyd and Parry, 1954).

The /

The concentrations reached within the cell were found to be very much in excess of those in plasma. Panteleeva (1950) stated that the exchange between erythrocytes and plasma was largely in the form of dehydroascorbic acid. If this is so, then the reports that ascorbic acid does not pass out of the erythrocyte are explained (Heinemann, 1940; Golden and Sargent, 1952).

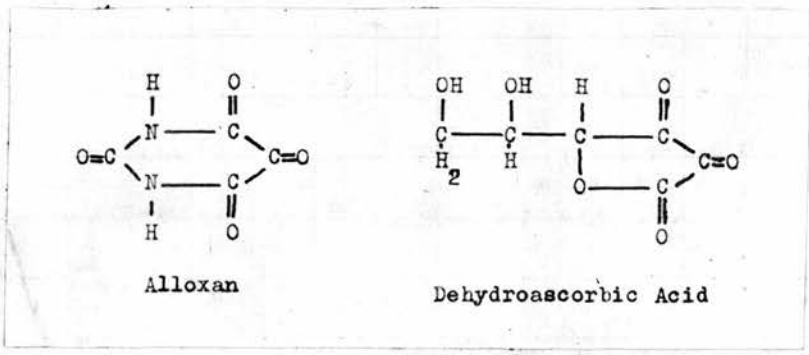
This capacity for the erythrocyte to reduce dehydroascorbic acid may be the explanation of the report by Langham (1950) that the intravenous injection of dehydroascorbic acid in the rabbit results in an increase in reduced ascorbic acid in the aqueous humour.

DEHYDROASCORBIC ACID DIABETES.

Several workers reported a lowered glucose tolerance in scurvy in experimental animals (Sigal and King, 1936; Banerjee and Ghosh, 1947), although this was not found in the case of experimental human scurvy reported by Grandon et al. (1940). An increase in tissue dehydroascorbic acid was reported in the same condition in guinea-pigs by Banerjee, Deb and Belavady (1952). This suggested a possible connection between diabetes and dehydroascorbic acid.

There are strong structural and chemical resemblances between dehydroascorbic acid and alloxan, a substance with recognised /

recognised diabetogenic action (Patterson, 1950).



Alloxan

Dehydroascorbic Acid

Both are relatively unstable at physiological pHs, both give the Strecker reaction with amino- acids, and both combine readily with sulphydryl compounds. This was demonstrated for dehydroascorbic acid by Drake, Smythe and King (1943) and for alloxan by Patterson, Lazarow and Levey (1949).

Alloxan diabetes has been studied and confirmed by very many workers in several species of animals since the original report of the production of diabetes in rabbits by Dunn, Sheehan and McLetchie (1943). The diabetes is a result of the necrotic action of alloxan on the β-cells of the Islets of Langerhans. The mechanism of the action is as yet uncertain. Brückmann and Wertheimer (1947) and also Lazarow (1949) have considered this, and although the hypotheses proposed by both groups of workers are attractive, there is, at present, no means of deciding which, if either, is /

is correct. It does appear that glutathione immobilisation is a factor in the production of the diabetic condition.

The resemblance of dehydroascorbic acid to alloxan, the lowered glucose tolerance and accompanying increase in tissue dehydroascorbic acid in scurvy were all reasons for interest in the results obtained by Patterson (1949) on treatment of rats with dehydroascorbic acid. He succeeded in producing permanent diabetes in rats by injecting dehydroascorbic acid. The diabetic condition was very closely parallel in histological, biochemical and physiological symptoms, complications, and response to treatment to that produced by alloxan (Patterson and Lazarow, 1950; Patterson, 1951). Whether dehydroascorbic acid can produce diabetes in other species was less certain. Princiotto (1951) reported diabetes in rabbits, following dehydroascorbic acid injection, but Banerjee, Belavady and Mukherjee (1953) have not been able to repeat this work.

The use of alloxan, with a certain amount of success, in the treatment of pathological hypoglycaemic conditions in humans, has been reported in a few instances (Talbot, Crawford and Bailey, 1948; Brunschwig, Allen, Owens and Thornton, 1944; Conn and Hinerman, 1948). However, the extreme toxicity of the drug, due to its action in producing renal failure will surely restrict its clinical use.

The /

The parallel between alloxan and dehydroascorbic acid seems sufficiently great to warrant the speculation that dehydroascorbic acid may be a factor in the aetiology of human diabetes mellitus.

Clayton et al. (1954) published results of the injection of dehydroascorbic acid methanolate in human subjects. This appears to be the first account in the literature of the injection of dehydroascorbic acid into humans. These authors did not, however, investigate the glucose tolerance of the subjects after the injection. They recommended caution in the use of dehydroascorbic acid in human subjects, in view of possible liver damage, such as they found in the guinea-pig. Patients who received injections of dehydro-ascorbic acid complained of local pain at the site of the injection, another reason to restrict the use of the oxidised product in experiments on humans.

Despite the obvious undesirability of injection of dehydroascorbic acid into humans, it seems of paramount importance to investigate the possible part that the vitamin derivative plays in diabetes mellitus. Indirect evidence may be important in this, with studies in vitro on human blood.

AIMS /

AIMS

The purpose of this investigation was the identification of the factors responsible for the regulation of the dehydroascorbic acid content of human blood. In the course of the study, a method was devised by means of which it was hoped to supply further evidence of the existence of dehydroascorbic acid in human blood plasma.

Blood from normal subjects, and from patients with pathological conditions, was examined with regard to the response to exogenous dehydroascorbic acid. Investigations in vivo and in isolated specimens of blood were carried out. It was hoped to establish the identity of the regulating factors, and the mechanism of their action in human blood.

METHODS

METHODS

Note: Throughout this section and the succeeding ones, it has been found convenient to use the following abbreviations.

AA = Ascorbic acid = "Reduced" ascorbic acid

DHA = Dehydroascorbic acid

DGA = Diketogulonic acid

Tot. AA = Total ascorbic acid = AA + DHA + DGA

GSH = Reduced glutathione

GSSG = Oxidised glutathione

1. ESTIMATION OF ASCORBIC ACID.

a. General Introduction.

Many methods of estimating ascorbic acid in pure solutions and in various biological materials have been proposed. These methods involve the use of such widely differing reagents as peri-naphthindanetrione hydrate, potassium iodate and $\alpha-\alpha'$dipyridyl. Most workers have, however, limited themselves to one or other of two basic techniques, or to the many modifications of these techniques which have been proposed at various times.

One method is based on the extreme ease of oxidation of ascorbic acid, and the other on the capacity of its two primary oxidation products, dehydroascorbic acid, DHA, and diketogulonic /

diketogulonic acid, DGA, to form a stable, characteristic hydrazone with 2:4 dinitrophenylhydrazine. The first method is used to estimate the reduced form of the vitamin, AA, and for ease of reference is termed the "indophenol" value; the second technique measures total ascorbic acid, Tot. AA, namely ascorbic acid, together with dehydroascorbic acid and diketogulonic acid. This is termed the "Roe" value. The concentration of oxidised ascorbic acid in the material under examination is determined by subtraction of the result obtained using the first estimation procedure from that obtained by use of the second.

$$DHA + DGA = \text{Tot. AA} - AA$$

b. Oxidation-Reduction Method.

i. Introduction.

The most commonly used reagents in this method are two redox indicators. Tillmans (1930) introduced the use of 2:6 dichlorphenolindophenol; the use of methylene blue was first advocated by Martini and Bonsignore (1934). Despite the greater sensitivity of the latter, indophenol is the preferable reagent, since it is more easily manipulated and is not light-sensitive, as is methylene blue. The method used in the investigations reported here is that described by Stewart, Horn and Robson (1953); a modified form of the method proposed by Bessey (1938) is also used for much of the experimental work. This incorporates sodium citrate as buffering /

buffering agent, a procedure found to be more convenient than the use of acetate, recommended by Mindlin and Butler (1938).

When the dye is added to an ascorbic acid solution, the pink colour normally exhibited in acid media is discharged. The discharge of the colour follows Beer's Law. This has been adapted for spectrophotometric estimations by adding excess dye to the ascorbic acid solution and then measuring the residual colour in the solution. Other substances cause fading of indophenol, but the decolorisation so produced takes place at a slower rate than that due to ascorbic acid. Reading of the residual colour at a fixed interval after the addition of the dye reduces the magnitude of the errors due to the presence of other substances reducing indophenol. The fading due to other substances is very much reduced if the hydrogen ion concentration of the solution is adjusted to a pH value between 3 and 4. By adjusting the pH to within this range with sodium citrate, the need to read the residual colour within the fixed interval is not so great, and so technical manipulations are easier. For convenience, the procedure in which sodium citrate is used is termed the "buffered" estimation method. The "unbuffered" estimation method is that in which indophenol is added directly to the acid filtrate of tissue, and the residual colour is read at a fixed interval after mixing.

ii. Method /

ii. Method Used in Estimation of Ascorbic Acid in Aqueous Solution, Serum and Plasma.

Reagents:

3% (w/v) metaphosphoric acid. This was freshly made for each series of estimations.

Dichlorphenolindophenol solution. This was prepared by dissolving a "Dichlorphenolindophenol B.D.H." tablet in water, filtering and making the volume up to 50 ml.

10% (w/v) sodium citrate.

5% (w/v) sodium citrate.

100mg./100ml. ascorbic acid.

A standard curve was prepared from dilutions of the ascorbic acid solution. The most convenient dilutions were found to be 0, 0.25, 0.50, 1.00, 1.50 and 2.00mg./100ml. ascorbic acid. These standard solutions were submitted to the same process as that outlined below for plasma, as rapidly as possible after the preparation of the original 100mg./100ml. solution.

Procedure:

4.0ml. plasma, serum or aqueous solution were gently mixed by inversion with 6.0ml. metaphosphoric acid, centrifuged and filtered. In order to obtain a clear filtrate, Whatman No. 42 paper was sometimes needed for filtration.

In the "unbuffered" estimation method, 2.0ml. of the plasma filtrate or standard solution-acid mixture were mixed with /

Table 1

Standard curve for "unbuffered" indophenol method for reduced Ascorbic acid

Ascorbic acid (mg./100 ml.)	Transmission reading (520 mμ)
0.25	63.0
0.50	65.2
1.00	73.0
1.50	82.0
2.00	91.2

Figure 1

Standard curve for "unbuffered" indophenol method
for reduced Ascorbic acid

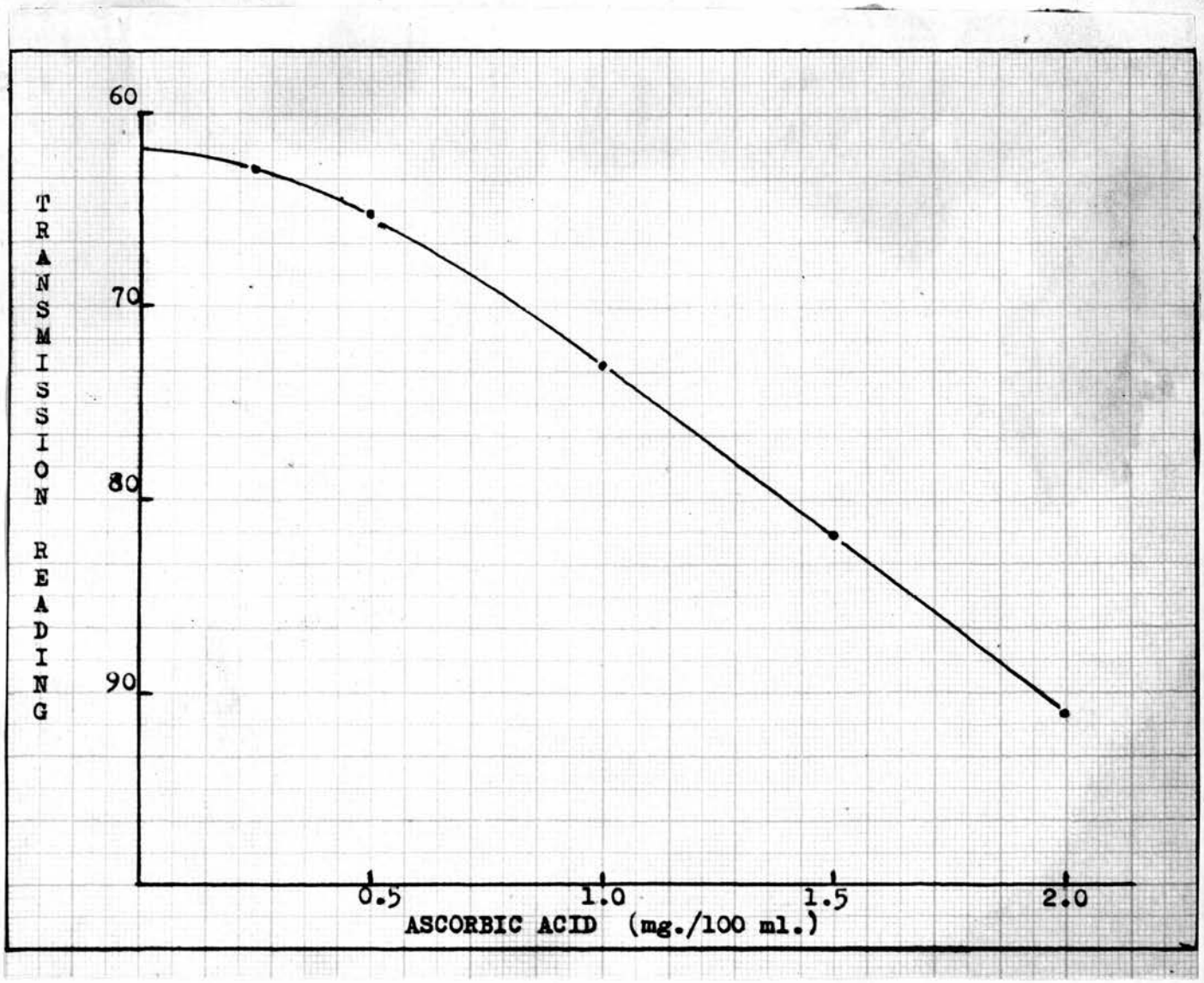


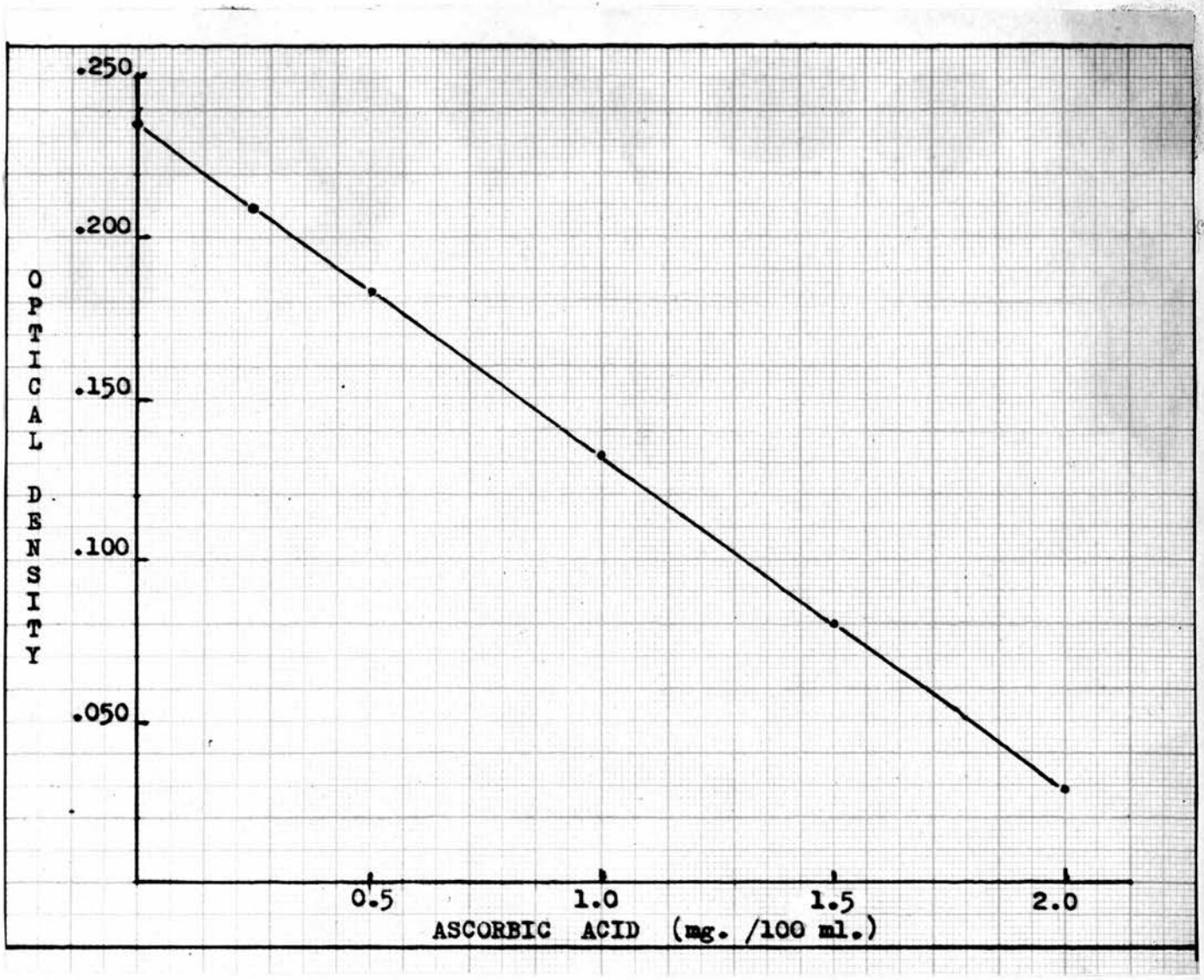
Table 2

Standard curve for "buffered" indophenol method
for reduced Ascorbic acid

Ascorbic acid (mg./100 ml.)	Optical Density Reading (520 mμ)	Blank	Corrected Reading.
0.00	.282	.046	.236
0.25	.255		.209
0.50	.229		.183
1.00	.178		.132
1.50	.126		.080
2.00	.075		.029

Figure 2

Standard curve for "buffered" indophenol method
for reduced Ascorbic acid



with 1.0ml. 2:6 dichlorphenolindophenol solution and the residual pink colour read 30 seconds later at 520mμ. in a Unicam SP 500 or SP 600 spectrophotometer. The filtrate was read within 3-4 hours after preparation, otherwise decay occurred, with subsequent errors in the estimation. Reading the standard solution filtrates against the indophenol solution resulted in the "standardisation" of the indophenol. The use of the standardised indophenol solution with the plasma filtrates permitted immediate estimation of the ascorbic acid concentration of the latter. Table 1 and Figure 1 show the standard curve for this method.

Using the "buffered" estimation method, 2.0ml. of the standard solution acid filtrate were mixed with 0.5ml. 10% sodium citrate solution before addition of indophenol. For plasma, 2.0ml. of plasma filtrate were mixed with 0.5ml. 5% sodium citrate and then indophenol was added. A pink colour of considerable stability was obtained in this way, and this could be read with accuracy at least two minutes after the addition of the indophenol. A correction for turbidity was made by decolorising all the solutions completely after they had been read, and subtracting the blank values so obtained from the original reading. The standard curve for the "buffered" method is shown in Table 2 and Figure 2.

The reproducibility of the results was tested. The mean result when 15 "unbuffered" determinations were carried out /

out on a specimen of plasma for AA, was 0.69±0.02mg./100ml., the range being 0.67-0.71mg./100ml. Recovery of added AA was 100%. Similar results were obtained for the "buffered" technique.

iii. Ascorbic Acid in Whole Blood.

Introduction:

Precipitation of whole blood with either metaphosphoric acid or trichloracetic acid, the precipitants normally employed in ascorbic acid estimation, resulted in lysis and release of haemoglobin. Lemberg et al. (1939) showed that there is a coupled oxidation of haemoglobin and ascorbic acid in the presence of cupric ions. AA estimations in lysed blood, in which Cu^{++} ions from plasma were present, were therefore invalid. These authors demonstrated that carbon monoxide saturation prevented this oxidation, and this resulted in the suggestion of Fujita, Ebihara and Numata (1939) that whole blood should be saturated with carbon monoxide prior to the estimation of ascorbic acid. This was developed into a method for estimation of AA in whole blood by Butler and Cushman (1940).

Method Used in Estimation of Ascorbic Acid in Whole Blood:

A modified version of the method of Butler and Cushman (1940) has been used in this work. Carbon monoxide was generated by the action of sulphuric acid on dry sodium formate and the soda- and water-washed gas was bubbled into a /

a tonometer containing the specimen of blood. The tonometer was rolled on its side during the procedure to aid thorough saturation. The length of time required for the passage of the gas was found to depend on the quantity of blood, but it was usually found adequate to allow 10 minutes for every 20ml. blood.

Reagents:

6% (w/v) metaphosphoric acid. This was freshly made.

Dichlorphenolindophenol solution.

5% (w/v) sodium citrate.

Procedure:

6.0ml. metaphosphoric acid were added to 4.0ml. CO--saturated blood and mixed thoroughly with a glass stirring rod to ensure complete protein precipitation. After standing 15 minutes, the mixture was centrifuged and filtered through a Whatman No. 42 paper. Estimation was carried out exactly as in the "buffered" method for ascorbic acid in plasma. The reproducibility on ten determinations was determined. The range was found to be 1.23-1.40mg./100ml., with a mean value of 1.31±0.06mg./100ml. Recovery of added AA was 99%.

iv. Ascorbic Acid in Erythrocytes.

Introduction:

A completely satisfactory method for estimating ascorbic acid in erythrocytes was not successfully devised, unless the concentration of reduced ascorbic acid was very high. Most work /

work done in the course of this study involved estimations on erythrocytes with such concentrations, and for these a satisfactory procedure was developed.

Method Used in Estimation of Ascorbic Acid in Erythrocytes:

Reagents:

6% (w/v) metaphosphoric acid. This was freshly made for each set of estimations.

Dichlorphenolindophenol solution.

2.5% (w/v) sodium citrate.

Procedure:

1.0ml. erythrocytes, previously saturated with carbon monoxide, was transferred by Pasteur pipette to a graduated centrifuge tube and precipitation was carried out in this tube with 6.0ml. metaphosphoric acid. Thorough stirring resulted in complete precipitation of the proteins; after standing 15 minutes, the mixture was diluted with 3.0ml. water to bring the volume to 10.0ml. This results in a 1 in 4 dilution of the ascorbic acid. If the saturation with carbon monoxide was complete, the precipitate was a red-brown colour, whereas a precipitate with a dark brown colour was produced with erythrocytes previously untreated with CO.

At the concentration levels of ascorbic acid investigated in this work, it was often found necessary to dilute the /

the filtrate obtained from the above precipitation to an even greater extent. This was done in the cuvette prior to reading in the spectrophotometer, to give finally a 1 in 16 dilution. In such circumstances, 2.5% sodium citrate proved to be the most suitable buffer concentration.

The reproducibility with cells diluted 1 in 4 for 10 determinations was found to be good. The range was 0.80-0.94mg./100ml. with a mean of 0.87±0.05mg./100ml.

v. Estimation of Ascorbic Acid in Plasma and Erythrocytes of a Blood Specimen.

The specimen of blood was saturated with CO and then centrifuged for 15 minutes. The plasma was separated, and the requisite amount of erythrocytes transferred to a graduated centrifuge tube. Precipitation of the proteins in both components was carried out as soon as possible after this. It was found that the process normally took 20 minutes to complete from the time centrifuging was begun until the proteins were precipitated.

For ease, the AA concentrations determined by this method are termed alternatively "indophenol values" for AA in the course of this report.

c. Use of Hydrogen Sulphide.

i. Introduction.

Hydrogen sulphide reduction has been used for many years/

years in the estimation of dehydroascorbic acid concentrations in tissues and biological extracts. Tillmans et al. (1932a) first applied it to lemon juice that had been oxidised with iodine, and showed the restoration of the capacity of the juice to reduce indophenol. Van Eekelen and her co-workers (van Eekelen et al., 1933) applied the treatment to trichloracetic acid filtrates of blood before testing for the presence of the vitamin. Subsequently, Emmerie and van Eekelen (1934) used H_2S treatment in the course of their method for estimating the vitamin in blood. The reducing action of H_2S on oxidised lemon juice was confirmed by Johnson (1933). He further pointed out the difficulty of removing the excess H_2S completely from such fruit juices. He also reported the presence of traces of gas even after 5 or 6 hours of treatment with wet nitrogen. He found, nevertheless, that after three hours' treatment with nitrogen, the amount of H_2S left was insufficient to affect his estimations.

Hydrogen sulphide reduction has been widely used to estimate the DHA concentration in tissues since 1933, but there has been a tendency to underestimate the probable errors attendant on its use. Apart from the difficulty of removing the last traces of the gas, it is a relatively unspecific reducing agent and its use may result in interference from non-vitamin C substances (King, 1941).

It /

It is difficult to assess the results obtained by different groups of workers, owing to the wide variation in the conditions used. There is little agreement on the length of time necessary for the passage of H_2S through the filtrate. Authors also differ about the hydrogen ion concentration of the filtrate, as well as the method used and the length of time taken to remove excess H_2S.

Levenson, Rosen and Hitchings (1951) investigated the reduction of DHA with H_2S, and they claimed good recovery from iodine-oxidised ascorbic acid solution in glycine-HCl buffer. They recommended H_2S treatment for 10 minutes at pH 3.19 and 37°; they claimed 94% recovery of AA under these circumstances. They gave no indication, however, of the length of time for which they passed nitrogen to remove excess H_2S.

The method employed in this laboratory was substantially that of Hochberg, Melnick and Oser (1944).

ii. Method.

Reagents:

Antimony trisulphide, precipitated from tartar emetic.

2N HCl

Reagents for "buffered" ascorbic acid method.

Procedure:

A 5.0ml. portion of the plasma filtrate, prepared for AA estimation, as described above, was buffered to pH 3.0-3.5 with /

with sodium citrate solution. 1-2 drops of caprylic alcohol were added to prevent frothing. Pure H_2S, generated by heating antimony trisulphide with dilute HCl, was bubbled through for 10 minutes at 37°C. Wet nitrogen was then passed through for 3 hours and the extract afterwards filtered to remove any sulphur that might have accumulated. Spectrophotometric examination with indophenol was then carried out in the same way as described for the "buffered" estimation for AA.

Interpretation of the results obtained by H_2S treatment was always cautious owing to the difficulty of removing the last interfering traces of the gas, and also to the unspecific reducing action of hydrogen sulphide.

d. Roe Method for "Total Ascorbic Acid".

i. Introduction.

This method is based on the formation of dinitrophenylhydrazone of the primary oxidation product of ascorbic acid. All ascorbic acid in the specimen being examined is oxidised with "norit" charcoal and, together with any pre-formed oxidised ascorbic acid, is converted to the dinitrophenylhydrazone by treatment with dinitrophenylhydrazine. According to Penney and Zilva (1943), the open-chain oxidation product diketogulonic acid (DGA) is the substance which reacts with the dinitrophenylhydrazine: the conditions of the /

the reaction favour the rapid conversion of the lactone, DHA, to its hydrated form, DGA. The concentration of phenylhydrazone formed is estimated by photometric measurement of the intensity of a red-brown colour produced on the addition of sulphuric acid. Penney and Zilva (1945) reported that reductones and reductic acid interfere with the estimation and the original authors, Roe and Keuther (1943) state that aldehydes, ketones and high concentrations of sugars introduce a positive error. It is, however, believed that these are relatively rare sources of error in human blood.

The results reported here as "total ascorbic acid" (Tot. AA) have all been obtained using the method described by Roe and Keuther (1943). It can be applied directly to all cellular components of blood, and to plasma equally satisfactorily since the oxidation of ascorbic acid on lysis of the blood does not invalidate the method.

ii. Method Used in Estimation of Total Ascorbic Acid.

Reagents:

6% (w/v) trichloracetic acid.

2:4dinitrophenylhydrazine. 2g. 2:4 dinitrophenylhydrazine dissolved in 100ml. 9N H_2SO_4. Filtered. Stored at 5 C. Renewed every 2 months.

"Norit" charcoal. 200g. "norit" charcoal boiled with 1 litre 10% (w/v) HCl. Filtered with suction. Washed with water until washings give a negative test for ferric iron. /

iron. Dried at 110-120°C for 12-16 hours.

Thiourea. 10g. thiourea dissolved in 100ml. 50% (v/v) aqueous alcohol. This reduces $KMnO_4$ readily.

Sulphuric acid. 85% H_2SO_4; prepared by adding 900ml. conc. H_2SO_4 to 100ml. water.

100mg./100ml. ascorbic acid.

Procedure:

3.0ml. blood fraction or aqueous solution were mixed, with vigorous shaking, with 9.0ml. trichloracetic acid. After centrifuging, if necessary, the supernatant liquid was decanted off, and approximately 700mg. "norit" charcoal were added. This was shaken for 6 minutes in a mechanical shaker and then filtered through a Whatman No. 42 paper. 2.0ml. filtrate were then transferred to another tube and a drop of thiourea and 0.5ml. dinitrophenylhydrazine reagent added. This solution was incubated for 3 hours at 37°C. The tube was placed in an ice-bath and 2.5ml. 85% H_2SO_4 were added. H_2SO_4 was added slowly to prevent overheating in the tube. Care was also taken to prevent H_2SO_4 coming in contact with traces of thiourea which might have crystallised out on the side of the tube. If this did occur, the final solution was cloudy, and as a result photometric estimation was inaccurate.

After the solution and added sulphuric acid had been thoroughly mixed, the tube was allowed to stand at room temperature /

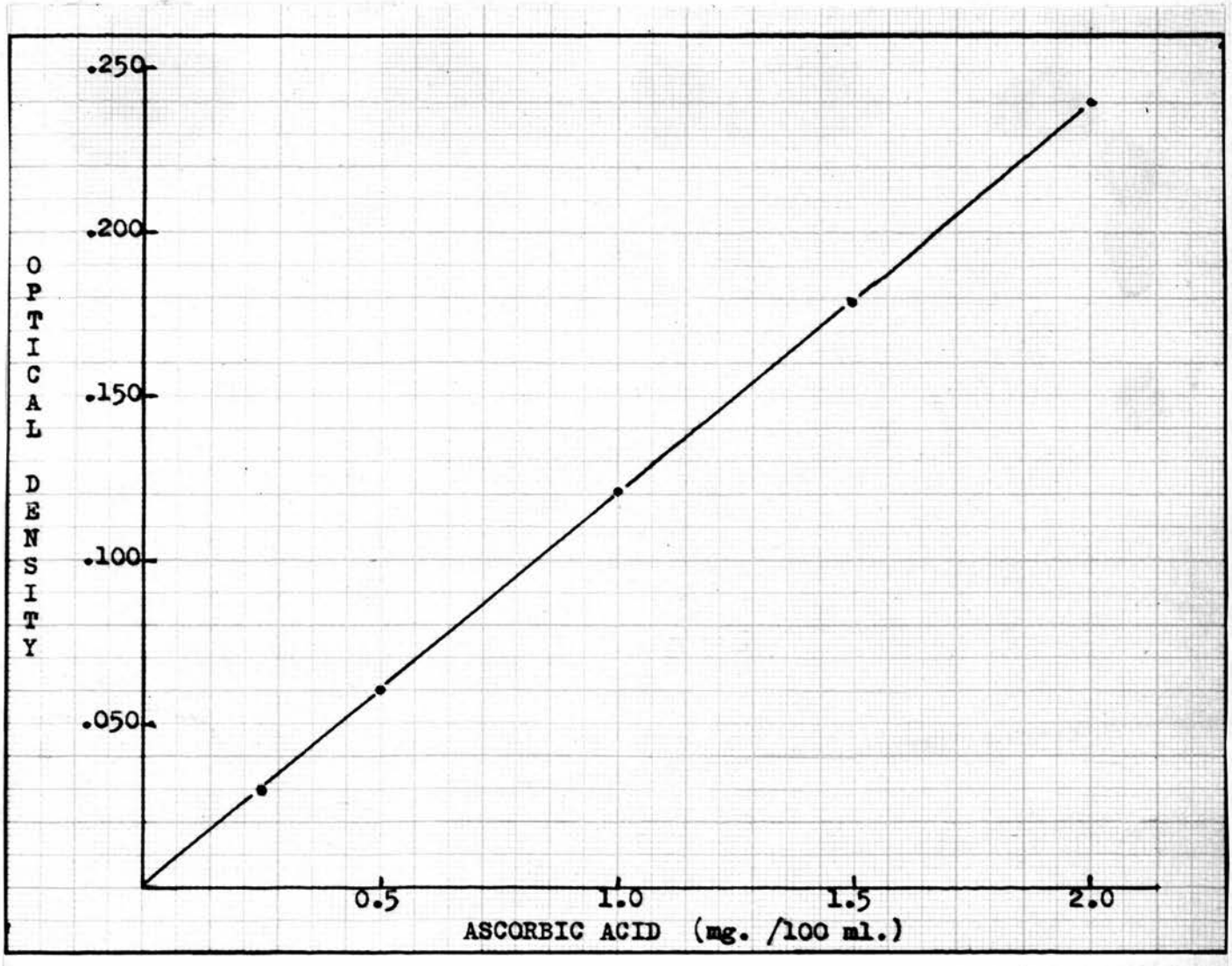
Table 3

Standard curve for Roe method
for total Ascorbic acid

Ascorbic Acid (mg./100 ml.)	Optical Density Reading (520 mμ)	Corrected Reading
0.00	.058	.000
0.25	.088	.030
0.50	.118	.060
1.00	.179	.121
1.50	.236	.178
2.00	.298	.240

Figure 3

Standard curve for Roe method
for total Ascorbic acid.



temperature for at least 30 minutes, before estimating the intensity of the brown colour. This was carried out photometrically using a Unicam SP 500 or SP 600 spectrophotometer, reading at 520mμ.

A standard curve of the brown colouration was prepared by diluting 100mg./100ml. ascorbic acid solution to give solutions of 0.25, 0.5, 1.0, 1.5 and 2.0mg./100ml. and submitting these dilute solutions to the procedure outlined above. Table 3 and Figure 3 show the standard curve.

In calculating the results, a blank value was subtracted from the readings for both standard solutions and test solutions. This blank was obtained by using 2.0ml. 4.5% CCl_3COOH in the above procedure, in place of 2.0ml. of the standard or test. Small variations in the standard curve were observed at various times. Their cause was not ascertained, but in view of their occurrence a standard curve was prepared for each group of estimations.

Results obtained by this method were of good reproducibility for whole blood and for blood constituents. Estimations of concentrations greater than 0.25mg./100ml. were regarded as reliable.

Reproducibility in plasma: 18 determinations. Range, 0.42-0.51mg./100ml. Mean, 0.44±0.03mg./100ml. 100% recovery of added AA.

Reproducibility /

Reproducibility in blood: 10 determinations. Range, 0.48-0.64mg./100ml. Mean, 0.52±0.04mg./100ml. 100% recovery of added AA.

Reproducibility in erythrocytes: 15 determinations. Range, 0.72-0.85mg./100ml. Mean, 0.81±0.05mg./100ml.

2. ESTIMATION OF GLUTATHIONE.

a. Introduction.

In the course of the work for this thesis, it was necessary to estimate the concentration of glutathione (GSH) in certain experiments. The method used was that employed by Bhattacharya, Stewart and Robson (1955). This was a modification of the classical method of Woodward and Fry (1932).

The blood under examination was lysed with sulphosalicylic acid and the proteins simultaneously precipitated. The soluble sulphydryl compounds - in effect GSH and its oxidised form, GSSG - were released into solution and the precipitated protein was filtered off. The tripeptide was then estimated by iodate titration.

b. Method Used in Estimation of Glutathione.

Reagents:

M sulphosalicylic acid. 25% (w/v) sulphosalicylic acid.

3% (w/v) sulphosalicylic acid.

4% (w/v) sulphosalicylic acid

M /

M/1000 KIO_3. 9.2ml. M sulphosalicylic acid were diluted to 90ml. approximately. 1 ml. 0.1N KIO_3 was added and the volume made up to 100ml.

5% (w/v) KI.

1% (w/v) starch solution.

Procedure:

3.0ml. whole blood were slowly added dropwise, with shaking, to 12.0ml. 3% sulphosalicylic acid. Thorough shaking was followed by centrifugation. The mixture was then filtered through a Whatman No. 1 paper.

1.3ml. 4% sulphosalicylic acid and 1.3ml. KI were added to 5.0ml. filtrate. Just prior to titration, 3 drops of starch were added and the mixture titrated against M/1000 KIO_3. The iodate was run in from a micro-burette until a permanent, very faint blue colour was obtained.

Calculation:

$$\frac{\text{Burette reading}}{3.26} \times 100 = \text{mg.GSH/100ml. blood.}$$

$$\frac{\text{Burette reading}}{3.26} \times 10 = \text{mg.GSH/50ml. filtrate.}$$

3. PREPARATION OF DEHYDROASCORBIC ACID.

a. Introduction.

DHA solution was used in much of the experimental work for this thesis. A rapid reliable preparation was necessary and that proposed by Patterson (1950) proved satisfactory. This /

This was a modification of the method originally devised by Moll and Wieters (1936) and involved oxidation of an aqueous AA solution with quinone, the latter being dissolved in ether.

b. Method.

500mg. AA were dissolved in 10ml. water or isotonic physiological saline. This solution was shaken for 15 minutes with 15ml. ether, in which 330mg. freshly sublimed quinone were dissolved. After removal of the ether the aqueous solution was washed 5 times with 10ml. portions of ether, and then residual ether removed by reducing the pressure within the mixture. The DHA solution prepared in this way was compared with the solutions obtained by oxidising AA with iodine or bromine. Their relative stability and their response to H_2S reduction were considered. There was no difference in response between the preparations. On this basis, it was decided to use the quinone-oxidised preparation as the experimental material for this work.

RESULTS

RESULTS

PART I. THE EXISTENCE OF DEHYDROASCORBIC ACID IN HUMAN BLOOD PLASMA.

Introduction.

Estimations of dehydroascorbic acid, as distinct from reduced ascorbic acid, in human blood plasma, have always been based on the increase in the indophenol reading of the filtrate after H_2S reduction has been carried out. Since this method is of doubtful reliability due to the non-specific reducing capacity of H_2S, it was considered desirable to estimate dehydroascorbic acid by some other method.

It was decided to apply a technique devised by Mapson and Ingram (1951) for use in plant extracts, employing Esch. coli. A strain of this bacterium has been found capable of reducing DHA and a suspension of this strain has been used in this work. The bacterial suspension was incubated anaerobically and under strictly controlled conditions with the solution under examination; the reduction of DHA by the bacteria was stopped by adding metaphosphoric acid, and so the amount of AA produced could be estimated.

Method.

The suspension of Esch. coli was prepared as described by Mapson and Ingram (1951) from a dried sample of bacteria (NCTC 4450). Mapson and Ingram's (1951) estimation procedure was slightly modified and is described below.

Reagents: /

Reagents:

Buffer 2% (w/v) HPO_3, buffered to pH 6.2 with 2% (w/v) NaOH. The hydrogen ion concentration was determined using chlorophenol red and bromocresol purple as external indicators.

25% (w/v) glucose solution.

Esch. coli suspension.

20% (w/v) metaphosphoric acid. This was freshly prepared for each estimation.

Procedure:

A stream of wet nitrogen was passed through 5.00ml. HPO_3-NaOH buffer, and 0.25ml. glucose at 35°C for 10 minutes. A drop of caprylic alcohol was added to the mixture to prevent frothing. 0.75ml. Esch. coli suspension was then added, followed by 4.00ml. of the plasma or solution to be tested. Nitrogen was passed for 15 minutes more, the temperature being maintained at 35°C, and then the reaction was stopped by adding 1.00ml. 20% HPO_3 to the reaction tube. After centrifuging and filtering through a Whatman No. 42 paper, estimation by the indophenol method was carried out in the usual way. The results were multiplied by factor (11/10) to correct for the variation from the normal volume proportions.

The method was tested on aqueous solutions of DHA and recoveries were carried out on plasma.

Results. /

Table 4

The reduction of dehydroascorbic acid in plasma by treatment with Esch. coli suspension

a. "Buffered" series

Subject	Ascorbic acid "buffered" indophenol (mg./100 ml.)	Ascorbic acid Esch. coli treatment (mg./100 ml.)	Total Ascorbic acid (mg./100 ml.)
R.R.	1.05	1.16	1.17
S.B.	0.92	0.98	1.04
J.R.	1.14	1.28	1.32
M.G.	1.33	1.36	1.32
C.T.	1.02	1.04	1.16
Mean	1.09	1.16	1.20

b. "Unbuffered" series

Subject	Ascorbic acid "unbuffered" indophenol (mg./100 ml.)	Ascorbic acid Esch. coli treatment (mg./100 ml.)	Ascorbic acid H_2S treatment (mg./100 ml.)	Total Ascorbic acid (mg./100 ml.)
J.R.	0.98	1.01	0.94	1.00
D.	0.52	0.57	0.72	0.70
C.T.	0.25	0.46	0.40	0.44
R.R.	0.28	0.26	0.23	0.29
A.L.	1.23	1.37	1.35	1.41
L.T.	0.15	0.29	0.28	0.25
Mean	0.57	0.66	0.65	0.68

Results.

The method was applied to specimens of plasma from volunteers and the results obtained were compared with those for reduced AA and for Total AA. Some estimations were carried out using sodium citrate buffer and others without buffering the filtrates; in the latter series, estimations after H_2S treatment were also made. The results for both series are quoted in Table 4.

Discussion.

The mean difference between the Roe and indophenol values for the results quoted was 0.11mg./100ml. in both sets of analyses. There was an increase in the indophenol value after reduction of the plasma filtrate with H_2S, the level attained being that of the Roe value, if allowance was made for the errors involved in the estimation procedures. This suggested the difference between the two values was due to the presence of DHA. Similar results were regarded by Stewart, Horn and Robson (1953) as suggestive of the presence of DHA in plasma.

Treatment of the plasma with the Esch. coli preparation resulted in an increased indophenol value in nearly all the specimens examined. The increase was such that the indophenol and the Roe values were the same within experimental limits after treatment with the bacterial suspension. The Esch. coli preparation had a specific reducing action with regard /

regard to DHA. It seemed justifiable to attribute the reason for the increase in AA concentration after bacterial treatment to the reduction of DHA originally present in the specimen.

This interpretation of the results inferred the presence of some oxidised ascorbic acid in human blood plasma, in the form of DHA, and was in agreement with the results from H_2S treatment. It confirmed previous reports of the presence of oxidised ascorbic acid in blood plasma (Lloyd et al., 1945; Chen and Schuck, 1950; Davey et al., 1952; Banerjee and Belavady, 1953; Stewart et al., 1953; Linksweiler, 1954.)

It was thought that the increase in indophenol values after treatment with the suspension of Esch. coli might be caused by the buffering of the solution in the course of the estimation, and that it might not reflect an actual reduction of DHA. This is not so, however, as the increase in AA was found after bacterial treatment of the plasma even when the buffered indophenol estimation method was used. It therefore seemed a valid inference that DHA did occur to a slight extent in human blood plasma and was susceptible to reduction by Esch. coli with a consequent increase in the AA concentration in the plasma.

Several hundred analyses of specimens of plasma were carried out in the course of the experimental work for this thesis using indophenol and Roe methods. The majority of these /

these, from both healthy and ill subjects, were found to show the difference between indophenol and Roe values, attributed, for the reasons outlined above to the presence of DHA. In no case was the concentration of DHA greater than 0.25mg./100ml. plasma and in most cases it was in the range 0.05-0.15mg./100ml. plasma.

PART II. REDUCTION OF DEHYDROASCORBIC ACID IN HUMANS.

1. Reduction In Vivo.

Introduction.

Earlier workers reported that administration of oxidised orange juice resulted in an increase in tissue and urinary AA concentration in guinea-pigs and humans (Johnson and Zilva, 1934; Borsook et al., 1937; Todhunter et al., 1950; De Ritter et al., 1951.) Thus, the existence of a biological reduction mechanism for DHA was recognised. Experiments were carried out in the course of this work to confirm these findings, using DHA prepared in aqueous solution by quinone oxidation.

Procedure.

Solutions of DHA were given orally to 6 adult patients in the Royal Infirmary with various clinical conditions. 10ml. portions of blood were removed by venipuncture with paraffined syringes into oxalated tubes. The plasma ascorbic acid concentrations were determined at various intervals after feeding. Both AA and Total AA were estimated; the difference between the values was attributed to the presence of DHA. Determinations were made 30, 60, 120 and 180 minutes after giving the DHA in each case, and in some of the cases, the concentrations at 300 and 1440 minutes were also determined.

DHA /

Table 5

Oral administration of dehydroascorbic acid

Patient T. (Rheumatoid arthritis)

Time (mins.)	AA (mg./100 ml. plasma)	Total AA (mg./100 ml. plasma)	"DHA" (mg./100 ml. plasma)	$\frac{AA}{\text{Total AA}}$
0	0.60	0.60	0.00	1.00
475 mg. DHA fed				
30	0.59	0.60	0.01	1.00
60	0.95	1.04	0.09	0.91
120	0.95	1.04	0.09	0.91
180	0.88	1.00	0.12	0.88
300	0.79	1.01	0.22	0.79
1440	0.68	0.72	0.04	0.94

Patient B. (Normal)

0	0.48	0.58	0.10	0.83
475 mg. DHA fed				
30	2.34	2.55	0.21	0.92
60	2.54	2.55	0.01	1.00
120	2.50	2.50	0.00	1.00
180	2.00	2.10	0.10	0.95
300	1.88	2.10	0.32	0.90
1440	1.14	1.51	0.37	0.76

Figure 4

The effect of feeding Dehydroascorbic acid
on the plasma Ascorbic and Dehydroascorbic acids.

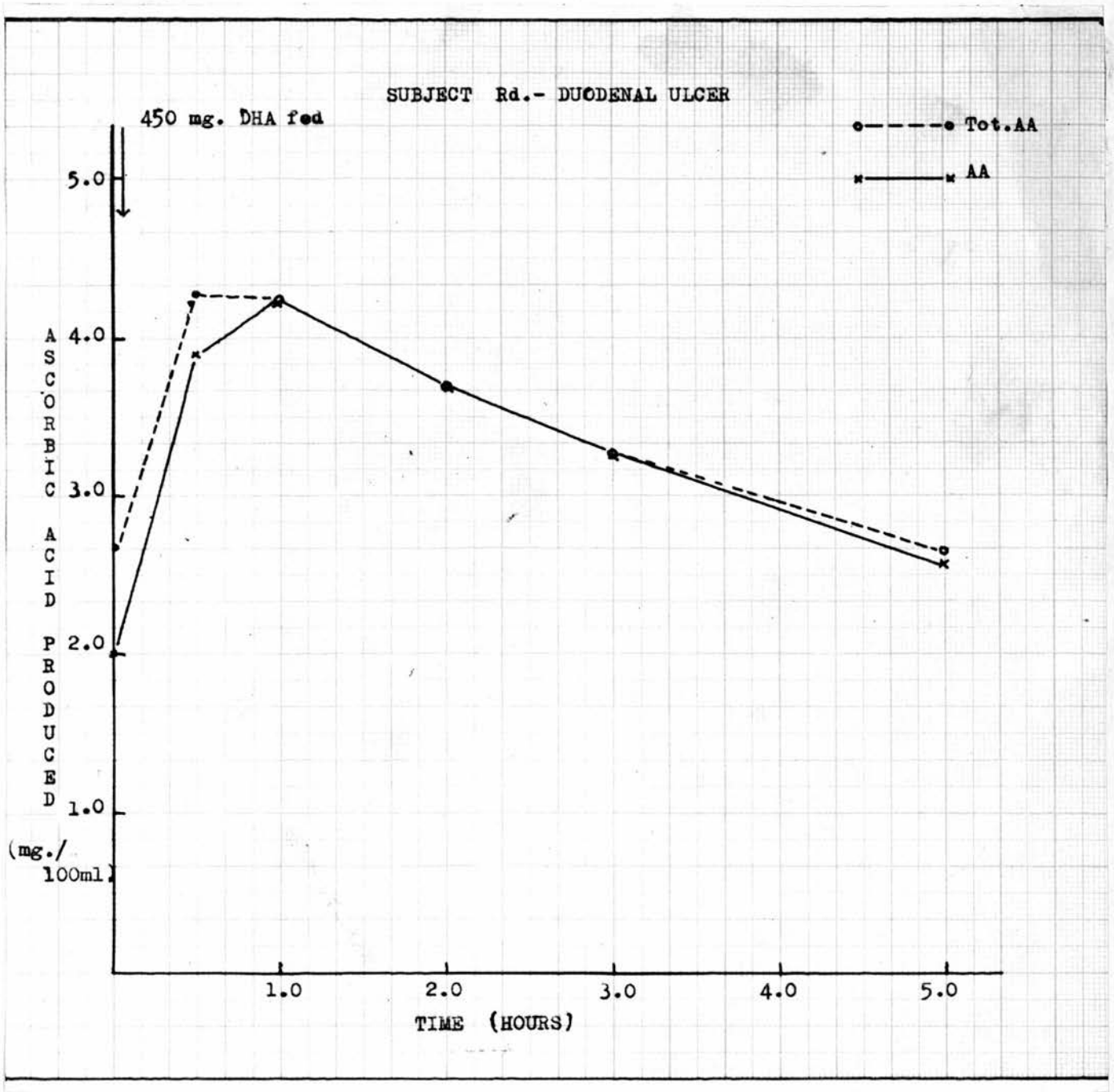


Table 6

Administration of dehydroascorbic acid *in vivo*

Patient	Condition	AA / Total AA 0'	DHA Dose mg.	AA / Total AA 30'	AA / Total AA 60'	AA / Total AA 120'	AA / Total AA 180'	AA / Total AA 300'	AA / Total AA 1440'
F.	Cirrhosis of liver.	1.00	470 fed	0.90	0.97	0.88	1.00	0.79	---
Rm.	Normal	0.85	250 fed	0.82	0.82	0.91	0.87	0.81	---
B.	Normal	0.83	475 fed	0.92	1.00	1.00	0.95	0.90	0.76
McD.	?Scurvy	-	470 fed	0.89	0.83	0.53	0.31	---	---
T.	Rheumatoid Arthritis	1.00	475 fed	1.00	0.91	0.91	0.88	0.79	0.94
Rd.	Duodenal ulcer.	0.75	450 fed	0.91	1.00	1.00	0.99	0.96	0.91
		0'		15'	75'	195'	1440'		
MacI.	Uraemia. Carcinoma of cervix.	0.39	16 by i.v. injection	0.66	0.36	0.14	0.09		

DHA was prepared by quinone oxidation of ascorbic acid and was given in aqueous solution.

Results.

The absolute changes in the plasma concentration of AA and Total AA differed from patient to patient. The general picture was, however, the same and a qualitative comparison was possible. Two typical cases are quoted in Table 5 and another represented in Figure 4.

Feeding DHA resulted in a sharp rise in both AA and Total AA in the plasma. The ratio AA:Total AA did not show any appreciable alteration at any stage after administration of the DHA. The ratios for all the cases examined are quoted in Table 6. In all cases except No. 4, McD., who was believed to be scorbutic, this ratio was never less than 0.76 and in most cases was considerably higher. The results for McD. were not reliable, since the methods of analyses were inaccurate at concentrations of ascorbic acid less than 0.25mg./100ml. and the plasma concentrations in this patient 120 and 180 minutes after administration of DHA were considerably less than this value.

Estimations for glutathione were carried out. In no case was there a significant alteration in the concentration at any time after feeding DHA.

Discussion.

Oral administration of DHA resulted in an increase in both /

both AA and Total AA in the plasma, such that there was no appreciable alteration in the ratio AA:Total AA. This indicated that little, if any, of the administered DHA appeared in the plasma unchanged, but was reduced to AA and was estimated in that form. The responsible reduction process was rapid since, 30 minutes after feeding, there was a marked increase in AA and Total AA and the highest concentrations were found 60 minutes after treatment.

The site of reduction of DHA and the extent of reduction could not be determined by these experiments on unsaturated subjects in vivo. Reduction might have occurred on the wall of the alimentary tract or in the blood itself or in both.

The increase in AA concentration in patient T, suffering from rheumatoid arthritis, after an oral dose of 475mg. DHA, was 0.45mg./100ml. in one hour (Table 5.) In the normal subject B, after the same dose, there was an increase of 2.06mg./100ml. in the same time. Patients suffering from rheumatoid arthritis have been reported to saturate less readily with vitamin C than do normals. This difference in response to the vitamin appeared to be reflected with regard to DHA in the results quoted in Table 5. Further consideration of the results did not, however, warrant this interpretation. None of the patients examined in this experiment had previously been saturated with ascorbic acid, nor was there any knowledge of their previous dietary régime or nutritional /

nutritional status.

The difference in response to administered DHA might therefore only indicate a difference in the previous nutritional condition of the two subjects with regard to ascorbic acid, and might bear no relation to their different physical conditions.

The risk attendant on the injection of DHA to humans was unknown, but it was considered inadvisable to inject it into a healthy individual, in view of its proved diabetogenic action in rats (Patterson, 1949). It was, however, administered by intravenous injection into one patient: this was a woman with advanced carcinoma of the cervix who was already moribund before the injection was given. The amount of DHA given was very much less than was used in the feeding experiments. DHA was prepared in a solution of isotonic saline and was filtered through a Seitz filter prior to injection. As in the case of scurvy previously discussed, some of the plasma values were suspect, since they were less than 0.25mg./100ml. Nevertheless, the ratio AA:Total AA appeared to be significantly less than in the patients to whom DHA was fed, suggesting that the DHA was appearing unchanged in the plasma. This disagreed with the findings of Clayton et al. (1954) who reported the appearance of AA in plasma after intravenous injection of commercially prepared DHA. The physical condition of the patient examined in this series was a possible /

possible reason for the impaired ability to reduce DHA.

2. Reduction in an Isolated Biological System.

(a) Existence of the Reduction:

Introduction.

Several workers (Borsook et al., 1937; Schultze et al., 1937; Kinkawa, 1944) have reported that isolated animal tissues and tissue breis reduced DHA. Whole blood and erythrocytes have also been found able to reduce DHA (Schultze et al., 1937; Panteleeva, 1950; Lloyd, 1951; Lloyd and Parry, 1954.) There are, however, no comprehensive accounts in the literature of erythrocyte or whole blood reduction of DHA. It was felt that further investigation of the phenomenon in humans was warranted, especially in the light of its reported diabetogenic action in other species (Patterson, 1949; Princiotto, 1951.)

Leucocytes and platelets are reported to have a high concentration of Total AA. Lowry, Bessey, Brock and Lopez (1946) observed concentrations of 12mg./100g. cells on a daily intake of 23mg. of vitamin C, while on an intake of 78mg./day they estimated the leucocyte concentration to be 25mg./100g. cells. There does not appear to be any report in the literature of work which differentiated AA and DHA concentrations in this fraction of the blood. Leucocytes were reported to be able to oxidise AA to DHA (Lloyd, 1951.)

In /

Table 7

Addition of dehydroascorbic acid to plasma and blood

Time (mins)	DHA addition[a] to plasma: plasma AA mg/100ml	DHA addition[a] to plasma: plasma Total AA mg/100ml	DHA addition[b] to whole blood: plasma AA mg/100ml	DHA addition[b] to whole blood: plasma Total AA mg/100ml
Control	0.42	0.46	0.18	0.21
	10 mg./100ml DHA added		10 mg./100ml DHA added	
0	0.42	10.00	0.60	6.75
120	1.00	10.00		
180			2.20	3.60
240	1.17	7.80		
360	1.38	4.62	3.80	4.54

In view of the high Total AA concentration and the reported oxidising capacity of this blood fraction, it was thought desirable to remove the leucocytes as far as possible from all specimens of blood before any investigations were carried out.

Procedure.

The effect of the addition of DHA to plasma and to blood was studied. The rate of decay was followed in both media. Estimations were carried out for AA and for Total AA in plasma; the effect of CO saturation was considered by comparing Total AA values after incubation with DHA in an aerobic atmosphere and in a CO atmosphere. CO saturation permitted AA determinations to be made on the erythrocytes. A comparison was made between the addition of AA and of DHA to whole blood.

Results.

Tables 7a and 7b show the plasma concentrations of AA and Total AA at different times after the addition of DHA to a) plasma and b) blood. The specimens were incubated at 37°C in all the estimations.

a) In plasma, Total AA measurements showed a quantitative recovery of the added DHA; AA estimations showed no increase over the control value, the analyses being carried out as soon after the addition of DHA to the plasma as possible. There was a 20% fall in the Total AA concentration in /

in 4 hours, and after 6 hours only 46% of the original DHA remained. Meanwhile, AA estimations showed 10% formation of a substance reacting towards indophenol in the same way as ascorbic acid. Biological assay would be necessary to establish if this substance was reduced ascorbic acid.

b) DHA was added to blood and the plasma separated off at intervals by taking a portion of the blood at the requisite time and centrifuging it. Analyses were then carried out on the separated plasma immediately. In the example quoted in Table 7b, only 40% of the DHA added was recovered from the plasma immediately after the addition of DHA.

[Note:

Packed cell volume of the specimen = 42%

$\therefore$ Complete recovery of added DHA = 17mg.Tot.AA/100ml. plasma

Actual concentration estimated = 6.75mg.Tot.AA/100 ml. plasma

$\therefore$ Recovery from plasma of added DHA = $\frac{6.75}{17} \times 100\%$

= 40%]

Six hours after the addition of DHA to the blood specimen the Total AA concentration in the plasma was 15% of the added DHA. AA estimations showed 38% formation of an indophenol--reducing substance six hours after the DHA addition.

It seemed improbable that 60% of the added DHA should have been destroyed immediately after its addition to blood. Total /

Table 8

Dehydroascorbic acid addition to whole blood

a. No carbon monoxide treatment.

PCV of specimen = 40%

Time mins.	plasma AA mg/100ml	plasma Total AA mg/100ml	cells Total AA mg/100ml	whole blood Total AA mg/100ml (by calcn.)
Dehydroascorbic acid added to give concentration 10 mg./100 ml. blood				
15	0.24	8.64	13.8	10.7
180	1.87	3.30	18.0	9.2
360	3.58	3.90	15.4	8.5

b. Carbon monoxide treatment.

PCV of specimen = 27%

Time mins	plasma AA mg/100ml	plasma Total AA mg/100ml	cells AA mg/100ml	cells Total AA mg/100ml	whole blood AA mg/100ml (by calculation.)	whole blood Total AA mg/100ml
Dehydroascorbic acid added to give concentration 10 mg./100 ml. blood						
30	0.30	4.26	20.6	25.7	5.8	10.1
120	1.20	2.79	21.6	20.9	6.7	7.7
180	1.64	2.55	21.6	20.5	7.0	7.4

Table 9

Comparison of the effect of ascorbic acid and dehydroascorbic acid addition to blood

Time	Supernatent			Cells			Whole Suspension (calc.)		
min.	AA mg/100ml	Total AA mg/100ml	DHA mg/100ml	AA mg/100ml	Total AA mg/100ml	DHA mg/100ml	AA mg/100ml	Total AA mg/100ml	DHA mg/100ml
	a. Ascorbic acid addition								
30	7.19	7.00	---	0.55	0.83	0.28	7.76	7.83	0.07
60	6.92	7.09	0.17	0.72	0.81	0.09	7.64	7.90	0.26
	b. Dehydroascorbic acid addition								
30	0.15	4.20	4.05	2.82	5.54	2.74	2.97	9.74	6.77
60	0.20	4.00	3.80	3.41	5.65	2.24	3.61	9.65	6.04

a. Percentage AA recovered as AA

30 min. = 99%

60 min. = 97%

b. Percentage DHA recovered as AA

30 min. = 31%

60 min. = 38%

Total AA determinations were therefore carried out on the cellular portion of blood after the addition of DHA. The results of one such experiment are detailed in Table 8a. The concentration was found to be high and, when expressed in terms of mg./100ml. blood on the basis of the PCV of the blood, gave, together with the concentration in the plasma, a quantitative recovery of the added DHA.

Carbon monoxide saturation of the blood prior to the addition of DHA made possible the estimation of AA in the cellular portion of the blood. Accordingly, a specimen of CO-saturated blood, to which DHA had been added, was analysed at intervals after the addition; AA and Total AA measurements were carried out in the plasma and the cells. The results are quoted in Table 8b. A high concentration of AA was found in the erythrocyte portion using this technique. The distribution of AA and Total AA was calculated and the results were expressed in mg./100ml. blood on the basis of the PCV.

The effect of the addition of ascorbic acid to blood was compared with that of DHA. In the examples quoted in Table 9, the examinations were carried out on red blood cell suspensions washed with isotonic saline to minimise any interference from endogenous AA and DHA. The difference in response to the two forms of vitamin C was marked and confirmed the report that, unlike the dehydro-form, AA did not /

not enter the erythrocyte (Heinemann, 1941; Golden and Sargent, 1952.)

Discussion.

It therefore appeared that, after its addition to blood from which as many leucocytes and platelets as possible had been removed, DHA passed rapidly into the erythrocytes and was there reduced to a considerable extent. The AA so produced then leaked slowly out of the cells. This confirmed the reports in the literature. The first step in the process, the passage of DHA into the cells, seemed to involve active transportation since it was so rapid, and also the concentration of ascorbic acid eventually attained within the cells was much greater than in the supporting plasma medium. The passage of AA out of the cells was, by contrast, probably controlled by a diffusion mechanism, since it occurred much more slowly. Alternatively, such passage of AA into the plasma might be the result of cellular damage, in view of the reports that AA did not pass out of the erythrocyte (Heinemann, 1941; Golden and Sargent, 1952.)

The passage of DHA into the erythrocyte was not influenced by the atmosphere in which the reaction occurred. The analyses for Total AA in Tables 8a and 8b showed clearly that a large proportion of the added DHA passed into the red blood cell. The appearance of AA in the plasma in the specimen examined without previous CO saturation was parallel to /

to a similar appearance in the CO-saturated specimen. CO saturation permitted technical analysis for AA in the red blood cell, but exerted no influence on the transport of DHA across the cell membrane.

Since the cellular portion of blood was seen to be the active element in the reduction of DHA, it was considered advisable to make comparisons using specimens with the same PCV. The PCV of all blood specimens examined was therefore adjusted to as near 30% as possible by centrifuging and removing either excess cells or plasma. 30% was an arbitrary choice for ease of calculation and also for convenience, as being attainable without undue waste of blood.

Comparison of results was only possible if the load of DHA was the same in all cases. The choice of a final DHA concentration of 10mg./100ml. blood was also fortuitous, but since it proved convenient in subsequent work, it was not thought necessary to use another concentration, except in certain cases. Indication of these will be given in the relevant sections.

(b) Reduction of DHA in Various Clinical Conditions:

Introduction.

There is an indication in the literature that tissue reduction of DHA is affected by the previous condition of the animal from which the tissues originate (Schroll, 1938; Vinokurov and Silakova, 1944; Matusis, 1951; Parrot and Gazave, /

Gazave, 1951).

Comparison of the response in different specimens of blood to added DHA was therefore attempted. A series of adults in normal health were used as subjects; analyses were also carried out on several patients suffering from a variety of conditions. The choice of the latter series of conditions was determined by the supply of available patients. Another group of analyses was compiled using diabetic subjects. The production of diabetes in rats after DHA injection (Patterson, 1949) aroused interest in a possible relationship between DHA metabolism and human diabetes mellitus. If such a relationship existed, it was thought that the reduction of DHA by the blood of confirmed diabetic patients might be abnormal.

The reduction of DHA was recognised to be a reaction governed by the erythrocytes. It was therefore decided to investigate the reduction in a condition where erythrocyte metabolism was known to be grossly abnormal. Pernicious anaemia is such a condition and there is the additional factor that it responds clinically to treatment with vitamin B_{12} in a short time and in a marked fashion. Investigation of the reduction of DHA in blood from patients with pernicious anaemia, before and during vitamin B_{12} treatment was therefore carried out.

It /

It was originally intended to compare the rate of reduction in different specimens using standard experimental conditions. The PCV of all specimens was adjusted to as near 30% as possible, the specimens were all saturated with CO and incubated in an atmosphere of the gas at 37°C. The concentration of DHA in the blood was as near 10mg./100ml. blood as possible. Estimations in cells and plasma could not be carried out in less than 20 minutes after addition of the oxidised vitamin for technical reasons and it was found in the large majority of cases, that reduction of DHA had gone to its fullest extent in that time. Comparison of the initial rate of reduction thus proved impracticable.

Comparison of absolute levels of ascorbic acid in cells and plasma was not satisfactory. The reduction of DHA was a property of the erythrocytes and the concentration of ascorbic acid attained in this fraction after adding DHA to blood was very high. Small variations in the erythrocyte concentration in different specimens of blood introduced large variations in the concentration of both forms of ascorbic acid in this erythrocyte fraction. Small variations in the concentration of DHA added to different specimens were magnified by the factor 100/30 (ml. blood/ml. RBC) on passing into the erythrocytes.

The most satisfactory means of comparison was eventually found to be the "percentage reduction of DHA". This was defined /

Table 10

Addition of dehydroascorbic acid to blood from normal subjects

Subject	AA (mg./100ml. blood)			Total AA (mg./100ml. blood)			"DHA" (mg./100ml. blood)			Percentage Reduction of DHA
	plasma	cells	whole blood	plasma	cells	whole blood	plasma	cells	whole blood	
J.O.♂	0.35	4.02	4.37	4.12	5.57	9.69	3.77	1.55	5.32	$\frac{4.37}{9.69} \times 100 = 46$
C.T.♀	0.34	4.33	4.67	4.57	5.64	10.21	4.23	1.31	5.54	$\frac{4.67}{10.21} \times 100 = 46$
E.B.♂	0.67	4.26	4.93	5.24	5.54	10.78	4.57	1.28	5.85	$\frac{4.93}{10.78} \times 100 = 46$
J.R.♂	0.19	4.77	4.96	4.65	5.91	10.56	4.46	1.14	5.60	$\frac{4.96}{10.56} \times 100 = 47$
J.McD.♂	0.65	3.76	4.41	4.69	4.72	9.41	4.04	0.96	5.00	$\frac{4.41}{9.41} \times 100 = 47$
F.M.♂	0.29	4.27	4.56	4.49	5.17	9.66	4.20	0.90	5.10	$\frac{4.56}{9.66} \times 100 = 47$
M.McL.♂	0.11	4.97	5.08	3.63	6.67	10.30	3.52	1.70	5.22	$\frac{5.08}{10.30} \times 100 = 49$
M.G.♀	0.33	4.83	5.16	4.66	5.54	10.20	4.33	0.71	5.04	$\frac{5.16}{10.20} \times 100 = 51$
A.T.♀	0.22	5.29	5.51	4.74	5.48	10.23	4.52	0.19	4.72	$\frac{5.51}{10.23} \times 100 = 54$
M.McD.♀	0.38	4.92	5.30	4.86	4.74	9.60	4.36	0.00	4.30	$\frac{5.30}{9.60} \times 100 = 55$
										MEAN = 48.8

defined as "the percentage of added DHA which is reduced to AA in whole blood, under standard conditions of PCV, initial DHA concentration and time, in an atmosphere of CO, at 37°C." Corrections could be made for variations in the concentration of erythrocytes, and of added DHA, using this means of comparison.

The method of calculation is shown in Appendix I.

The percentage reduction of DHA was measured 30 minutes after the addition of the dehydro-form. Technical manipulations prevented estimations being made in a shorter time after the DHA addition; decay of the added DHA was still negligible at this interval.

Procedure.

The blood, with the leucocytes removed as far as possible and the PCV adjusted to 30%, was saturated with CO. DHA was added to give a concentration as near 10mg./100ml. blood as possible. The blood was then incubated for 10 minutes in an atmosphere of CO at 37°C. It was centrifuged for 15 minutes in a MSE table centrifuge at speed 10. Precipitation of the plasma and erythrocyte proteins was carried out in the remaining 5 minutes.

Results.

Blood from a series of subjects in good health was analysed and the results are given in Table 10. The percentage reduction of DHA was calculated in each case, using the method /

Figure 5

The variation in the percentage reduction of Dehydroascorbic acid in blood from normal and pathological conditions.

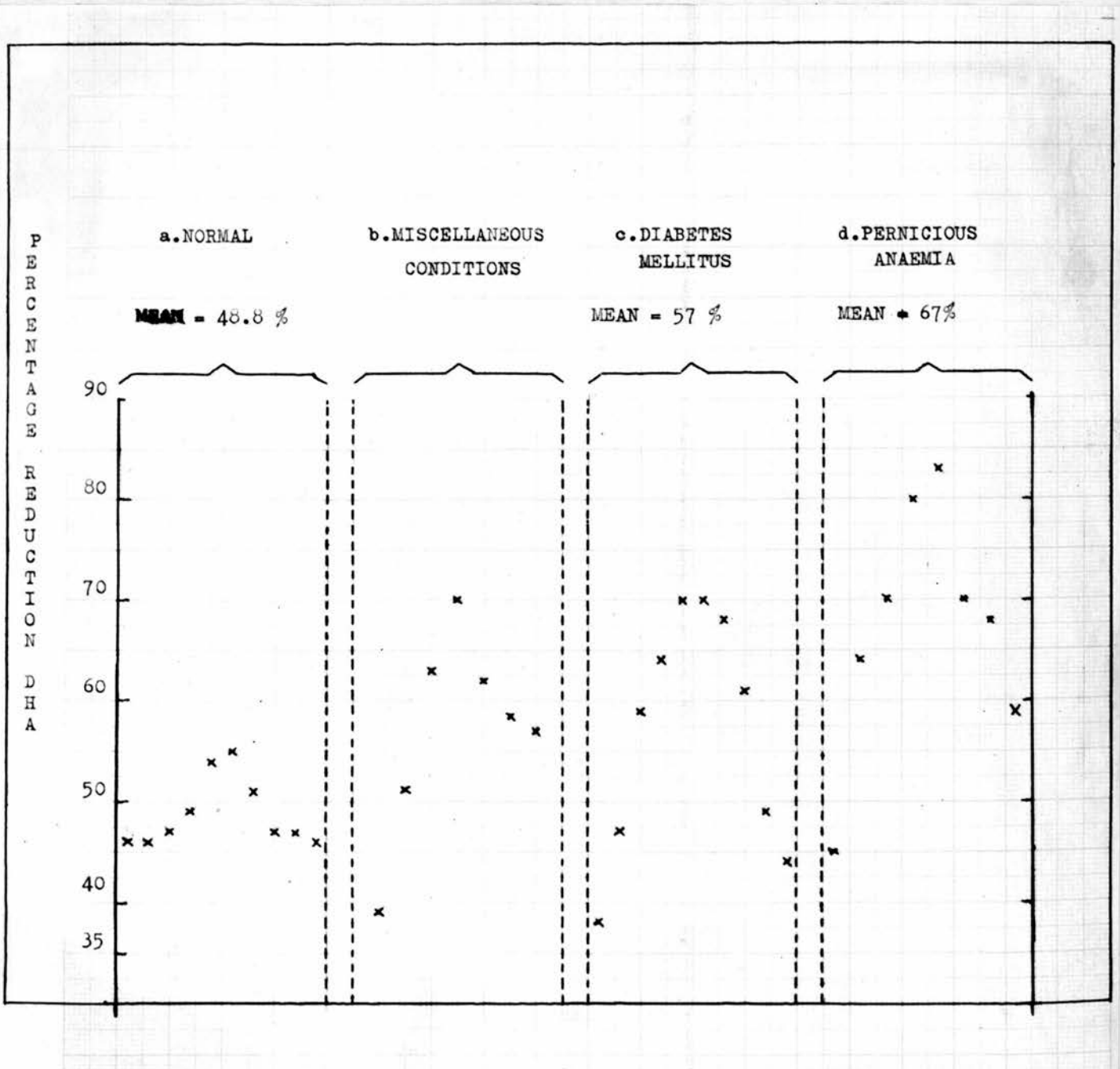


Table 11

Addition of dehydroascorbic acid to blood from patients with different pathological conditions

Subject	Clinical Details				Percentage Reduction of DHA
	Condition	Hb %	W.B.C.	B.S.R. mm/hr	
M.W.♀ 47 years.	Essential hypertension.	90	5,700	-----	62
D.R.♂ 32 years.	Haemochromatosis. No diabetes.	94	----	-----	51
W.C.♂ 15 years.	Duodenal ulcer.	74	----	-----	70
J.D.♂ 25 years.	Bronchial carcinoma.	108	5,600	15	58
G.McM.♀ 46 years.	Cardiac Neurosis. Hypertension.	102	7,000	1	63
J.McD.♂ 23 years.	Rheumatic fever.	96	10,400	53	39
M.B.♀ 47 years.	Mitral stenosis. Mitral incompetence.	85	6,000	8	57

Table 12

Addition of dehydroascorbic acid to blood from patients with diabetes mellitus

Subject	Clinical Details					Percentage Reduction of DHA
	Condition	Insulin	Hb %	W.B.C.	B.S.R. mm/hr	
J.D.♂ 73 years.	Diabetes. Cerebral thrombosis.	18 ZPI	94	9,000	3	70
K.G.♀ 30 years.	Diabetes.	48ZPI 16sol.	--	--	--	70
E.S.♀ 55 years.	Diabetes.	12ZPI 12sol.	--	--	--	68
E,M.♀ 49 years.	Diabetes.	40ZPI 36sol.	106	5,600	--	64
H.McE.♀ 75 years.	Diabetes.	None. Diet control.	106	6,200	--	61
H.W.♂ 77 years.	Diabetes.	24 IZS	--	--	--	59
M.F.♀ 55 years.	Diabetes.	44 IZS	100	8,400	45	49
A.A.♂ 24 years.	Diabetes.	72 IZS	106	--	--	47
J.P.♂ 28 years.	Diabetes. Tuberculosis.	52 ultra.	102	6,200	7	44
A.S.♂ 39 years.	Diabetes.	60 IZS	--	--	--	38
					Mean =	57

method outlined in Appendix I. The range of results for the percentage reduction was found to be narrow for this series of 10 people. Range = 46-55%. The mean value was found to be 48.8%. The scatter of results is shown in Figure 5a.

The percentage reduction of DHA in blood drawn from patients suffering from a variety of conditions was then considered. The results in Table 11 were obtained from examinations carried out on various unrelated conditions. One patient examined, J.McD., was suffering from rheumatic fever in which abnormal metabolism in the erythrocyte might have been expected. As far as is known, there was no RBC abnormality in any of the other conditions examined. The range of values for the percentage reduction was very much wider than that found for the normal subjects, as can be seen in Figure 5b.

10 diabetic patients were used as subjects and the percentage reduction of DHA for each specimen is quoted in Table 12. The scatter of values is shown in Figure 5c. The mean percentage reduction of DHA was found to be 57% for this group, and the range to be 38-70%.

The fourth group of patients examined were those suffering from pernicious anaemia. Only 8 subjects could be examined, since most patients admitted into hospital had previously been given emergency treatment and were therefore not /

Table 13

Addition of dehydroascorbic acid to blood from patients with pernicious anaemia

Subject	Clinical Details							Percentage Reduction of DHA		
	Condition	Time of estimation	Hb %	C.I.	W.B.C	R.B.C. mill.	Retic.	1.	2.	3.
A.M. ♂ 37 years.	Addisonian pernicious anaemia. Vitamin B_{12} treatment.	Admission	35	1.30	4,600	1.32	<1	83		
		Max respce.	--	--	--	--	15.0		65	
		Discharge	64	1.01	6,600	3.16	3.0			77
M.A. ♀ 58 years.	Addisonian pernicious anaemia. Blood transfusion. Vitamin B_{12} treatment.	Admission	40	--	--	--	<1	80		
		Max respce.	54	1.03	7,400	2.61	9.2		69	
		Discharge	77	1.01	5,600	3.81	<1			67
J.B. ♀ 62 years.	Addisonian pernicious anaemia. Vitamin B_{12} treatment.	Admission	67	1.47	5,600	2.28	<1	70		
		Max respce.	67	1.35	6,800	2.48	17 .1		67	
		Discharge	83	1.22	10,600	3.39	6.8			68
C.P. ♀ 63 years.	Addisonian pernicious anaemia. Vitamin B_{12} treatment.	Admission	59	1.39	4,000	2.12	2.3	70		
		Max respce.	69	1.19	6,800	2.89	10.9		62	
		Discharge	80	1.10	--	3.63	7.3			65
A.C. ♀ 61 years.	Addisonian pernicious anaemia. Vitamin B_{12} treatment.	Admission	68	1.21	4,400	2.79	1.0	68		
		Max respce.	85	1.18	6,000	3.72	6.0		72	
I.L. ♀ 52 years.	Addisonian pernicious anaemia. No Vitamin B_{12} treatment.	Admission	75	1.14	4,400	3.30	2.0	64		
		Discharge	75	1.14	5,600	3.28	<1			63
F.B. ♂ 78 years.	Addisonian pernicious anaemia. Vitamin B_{12} treatment.	Admission	30	1.16	10,000	1.29	<1	59		
		Max respce.	33	--	12,100	1.24	28.0		73	
		Discharge	60	0.94	8,400	--	--			69
J.W. ♂ 62 years.	Subacute combined degeneration. Vitamin B_{12} treatment.	Admission	97	1.31	5,400	3.68	<1	45		
		Discharge	94	1.04	8,600	4.51	1.0			70
							Mean =			67

not suitable for this survey. All the patients were examined prior to clinical treatment; the mean percentage reduction was found to be 67%, with a range 45-83%. The results are listed in Table 13 and Figure 5d. One subject, J.W., was not anaemic, but was included in this series because he was receiving vitamin B_{12} treatment. He was diagnosed to be suffering from subacute combined degeneration, a neurological condition usually associated with pernicious anaemia. He responded clinically to vitamin B_{12} therapy.

100γ of "Cytamen" was administered daily in most cases. Analyses were carried out after "Cytamen" treatment was commenced and the results quoted in column 2 of Table 13 are for the period when the clinical response was maximal. The maximal clinical response was assessed by the reticulocyte count. In one case, M.A., one pint of glucose citrate dextrose blood was transfused as a preliminary to other treatment. Vitamin B_{12} was then given as in the other cases. In the case of I.L., no treatment with the vitamin was used. Although the patient was diagnosed to be suffering from pernicious anaemia, sternal puncture showed a normoblastic marrow and she was therefore regarded as being in natural remission.

Each patient was examined a few days prior to discharge from hospital and the percentage reduction of DHA found in these estimations is quoted in column 3 of Table 13.

There /

There is no figure corresponding to a period of maximal response in the cases of J.W. and I.L., since the former showed no marked reticulocyte response, and the latter received no "Cytamen" treatment.

Discussion.

Interpretation of the results obtained with these four groups of subjects can only be tentative. The percentage reduction value of DHA for a normal subject appears to fall within a narrow range; age and sex do not appear to influence the value of the ratio.

Infections are possible reasons for a change in the reduction percentage. Yet, J.McD., Table 11, with a diagnosis of rheumatic fever, and on salicylate therapy, does not show a strikingly lowered percentage reduction. There are no grounds to regard the value, 41%, as abnormal. Abnormal values would not be surprising in conditions with an associated erythrocyte dysfunction. None of the conditions for which results are quoted in Table 11, however, are expected to show such a dysfunction (Duncan, 1952) yet the reduction percentage values are scattered over a much wider range than is found for healthy subjects. Complete haematological data was not obtainable for these subjects. There seems to be a tendency for the reduction percentage to be greater than normal as the percentage haemoglobin falls below the normal. At present this is only a tentative interpretation /

interpretation of the results.

Conclusions from the results for the series of diabetic patients are even less definite. In view of the action of injected DHA in rats, it seemed of interest to determine if an impaired erythrocyte reduction of DHA existed in human patients suffering from diabetes mellitus. A possible hypothesis is that such an impairment in reduction would result in an accumulation of DHA in the circulation and a consequent development of diabetes. Whether such a situation does exist is, of course, not known, but the results obtained for the percentage reduction of DHA in the blood of human diabetics make it seem very unlikely. In all but one case the results either lie within, or are higher than the normal range. The range of values is very much wider than that for the normal series. There does not appear to be any parallel between the severity of the diabetic condition, judged by the insulin dosage of the patient, and the value for the reduction percentage of DHA. Whether there is any relation between the latter and the state of insulin control is not certain, but there is no evidence to suggest that any of the patients were out of control at the time of the examinations. There is no inverse relationship to be observed between the haemoglobin concentration and the percentage reduction value for DHA in this group.

The haemoglobin concentration, or more accurately, the packed /

packed cell volume of the blood under examination appears, however, to be related inversely to the percentage reduction of DHA values in the cases of untreated pernicious anaemia examined. The severity of the disease, assessed by the lowering of the PCV, appears to be directly related to the percentage reduction. In some specimens of this group, a fall in the percentage reduction occurs at the period of maximal response, but this is not found with all the cases.

Examinations carried out prior to discharge of the patients from hospital showed that none of the specimens had attained a normal percentage reduction of DHA. There was no appreciable decrease in the percentage reduction corresponding to the marked increase in the PCV observed in all the anaemic patients receiving "Cytamen" therapy. There was no parallel between the haemoglobin concentration values and those for the DHA reduction percentage.

While the clinical response to vitamin B_{12} is observed in a very short time, usually 3-8 days after commencement of treatment (Duncan, 1952), the fundamental abnormality is not completely corrected for a considerably longer time. The life of a normal circulating erythrocyte is about 120 days. It is reported that in untreated pernicious anaemia there is a shortened RBC survival time which becomes normal once treatment is commenced (Singer, King and Robin, 1948.) It may be assumed that a large proportion of the erythrocytes circulating /

circulating in the body for at least a month after the commencement of treatment with vitamin B_{12} are erythrocytes which were in the circulation before treatment was commenced. Treatment with vitamin B_{12} promotes normal erythropoiesis, but has no effect on the RBCs already released into the circulation. These still influence the behaviour of the circulating blood. In view of this, an initial abnormally elevated percentage reduction for DHA may not be expected to fall to the normal range rapidly. In some specimens a fall in the extent of the reduction was observed after a period of treatment, but this decrease was never large. In case M.A., the transfusion of one pint of normal blood, and the resultant rapid lowering of the concentration of abnormal cells, was a possible contributory factor to the decrease in the percentage reduction in this subject.

All the estimations tabulated in Table 13 were therefore carried out while the erythrocyte conditions were still abnormal, although a marked clinical improvement had been observed. Determinations carried out after some months of vitamin B_{12} therapy should be of interest, as the gross abnormality responsible for the anaemic condition should by then be considerably, if not completely ameliorated. Such examinations have so far not been carried out, but it is hoped that they may be conducted in the near future. In cases of pernicious anaemia, which are in complete remission after /

after several months of vitamin B_{12} therapy, erythropoiesis should be normal as should be the circulating cells. It seems reasonable to expect in such cases that the percentage reduction of DHA should also be normal.

The conclusions to be drawn from the examination of the reduction percentage values of DHA in normal and pathological human blood seem to be as follows:

1) In blood from normal subjects in good health, there is a percentage reduction value for DHA of approximately 50%. The range is narrow.

2) A variety of completely unrelated conditions can cause an elevation in the percentage reduction value. In several diseases, the rise in this value appears to be related inversely to the packed cell volume or to the haemoglobin concentration of the blood under examination. This appears to be particularly marked in untreated Addisonian pernicious anaemia. Treatment with vitamin B_{12} appears to disrupt this relationship.

3) Haemoglobin concentration does not seem to be a controlling factor in the cases of diabetes examined. Neither the severity of the diabetic condition nor the state of insulin control appears to influence the level of reduction of DHA.

PART III. MECHANISM OF THE REDUCTION OF DHA BY HUMAN BLOOD.

Introduction.

The reduction of DHA by tissues has been recognised to be due to sulphydryl substances (Barron et al., 1936a; Borsook et al., 1937; Schultze et al., 1937). Schultze and his colleagues (Schultze et al., 1937) attributed the reduction of DHA in whole blood to the presence of GSH in that medium. Panteleeva (1950), Lloyd (1951) and Lloyd and Parry (1954) confirmed the reduction of DHA by blood but advanced no evidence about the identity of the hydrogen donor. Reports in the literature showed that the reduction in tissues did not fit the theoretical equation

$$DHA + 2GSH = AA + GSSG$$

and supplementary or alternative reduction mechanisms were postulated (Borsook et al., 1937; Kinkawa, 1944.) There does not appear to be any report of experimental investigation of the mechanism of the sulphydryl reduction of DHA in blood. An attempt has been made in the course of this study to identify and characterise the responsible mechanism. Certain information concerning it has been accumulated.

All analyses in this section were carried out using blood withdrawn from a patient with haemochromatosis, who was not, however, diabetic. This patient, D.R., whose blood was found to reduce DHA to the same extent as that of a normal subject (Table 11), reported to the Royal Infirmary for /

for weekly examination. At each visit one pint of blood was withdrawn by venesection using a heparinised outfit. The blood was stored at 4°C in a bottle containing heparin. No preservative was added. Determinations were never carried out on blood older than 7 days, in the course of these experiments.

In the investigations, DHA was added to various preparations of blood or blood constituents, using different experimental conditions which are detailed in the relevant sections. Unless otherwise stated, a concentration of 10mg.DHA/100ml. blood was used. The extent of reduction was evaluated by the amount of AA produced in the reaction.

1. Location of the Reducing Mechanism.

Introduction.

The work outlined in Part II makes it abundantly clear that the reduction of DHA in blood is accomplished by the erythrocytes. The effect of haemolysis of blood on the reduction was studied. Haemolysed blood was centrifuged and the supernatant was examined for its reducing capacity.

Procedure.

A specimen of blood was centrifuged, the leucocytes removed and the specimen saturated with CO. A portion was then haemolysed by shaking with saponin and the reduction of DHA in this portion was compared with that in an unhaemolysed portion /

Table 14

The addition of dehydroascorbic acid to haemolysed blood

a.

Specimen	AA produced (mg./100ml. blood)		
	15 min.	30 min.	60 min.
Whole blood.	4.20	4.15	4.10
Haemolysed blood.	2.60	3.60	4.05

b.

Specimen	AA produced (mg./100ml. blood)	
	30 min.	60 min.
Haemolysed blood.	1.42	1.50
Haemolysed, centrifuged blood.	1.36	1.42

portion of the same sample of blood. Table 14a shows the results obtained.

Another portion of the same sample of blood was centrifuged to remove erythrocyte stroma and the reduction of DHA was compared in the centrifuged and non-centrifuged haemolysed blood. Table 14b shows the extent of the reduction in the two portions.

Results.

Haemolysis was found to diminish the rate of the reduction of DHA but did not affect the extent of the reduction. The concentration of AA produced was unaltered by centrifugation of haemolysed blood. It was concluded that the reduction mechanism lay within the erythrocyte and not on the wall of the cell. Haemolysis caused dispersion of the reducing agent or agents and therefore slowed down the reduction but did not alter the concentration of DHA eventually reduced.

2. Extent of the Reduction.

Introduction.

The rate of reduction of added DHA within the RBC has been shown to be rapid. The extent of the reduction was found to vary, depending on the condition of the subject from whom the blood was drawn. Even in normal subjects, an individual variation was found in the amount of DHA reduced. This /

Table 15

Effect of increase in dehydroascorbic acid concentration on percentage reduction of dehydroascorbic acid

Time since last addition DHA (min.)	Concentration DHA (mg./100ml.)	Concentration AA (mg./100ml.)	Percentage Reduction of DHA
0	-	0.07	-
15	10	5.56	56
30	10	5.60	56
15	20	11.12	56
30	20	11.84	59
30	30	17.60	58
15	40	23.2	58
30	40	22.8	57
15	50	27.4	55

This suggested that the responsible mechanism became exhausted and could not carry the reduction to completion. A specimen of blood was examined to check the truth of this concept.

Procedure.

A specimen of blood was saturated with CO and DHA was then added to give a concentration of 10mg./100ml. blood. It was incubated at 37°C. When reduction was apparently complete, more DHA was added to raise the concentration of DHA in the blood to 20mg./100ml. After further estimations, subsequent increases in the DHA concentration were made to 30mg./100ml. and then to 40mg./100ml. and 50mg./100ml. The concentration of AA in the blood was estimated at each level of DHA concentration.

Results.

The results are shown in Table 15. It appeared that the absence of further reduction of DHA did not necessarily indicate that the reduction mechanism was exhausted. Rather, the reduction occurred to an extent characteristic of the blood under examination and the percentage of DHA reduced to AA remained constant for that blood, regardless of the initial concentration of the DHA added. This was true until high concentrations of DHA were reached.

3. Identity /

Table 16

The effect on the reduction of dehydroascorbic acid Produced by altering the -SH concentration

a.

	Concentration GSH (mg./100ml.)	Concentration AA produced (mg./100ml.)	
		Control	GSH supplemented
i	15	0.80	1.77
ii	20	1.37	1.97
iii	30	1.00	2.12

b.

Concentration GSH (mg./100ml.)	Concentration AA produced (mg./100ml.)	Concentration cysteineHCl (mg./100ml.)	Concentration AA produced (mg./100ml.)
40	8.5	20.5	8.1
30.7	4.4	15.8	4.5

c.

Concentration AA produced (mg./100ml.)	
Control	Egg albumin supplemented
1.18	1.33

d.

Concentration inhibitor	Concentration AA produced (mg./100ml.)	
	Control	Inhibitor added
0.01M CH_2ICOO'	2.30	0.35
0.01M CH_2ICOO'	3.44	0.29
0.005M CN'	4.2	3.2
0.05M CN'	2.6	0.4
0.01M CN'	3.2	indophenol fading

3. Identity of the Reducing Agent.

Introduction.

Evidence has been accumulated confirming the reports that DHA reduction was brought about with -SH groups as hydrogen donors. Addition of soluble -SH donors increased the extent of the reduction in blood. -SH inhibitors prevented the reduction taking place completely.

a. Procedure.

A specimen of haemolysed blood was saturated with CO and divided into two portions. Concentrated glutathione solution was added to one portion and the reduction of DHA solutions of the same concentration was determined in both portions, by estimating the AA produced after each specimen had been incubated at 37°C for one hour.

Results.

Results are detailed in Table 16a. The addition of GSH to blood increased the extent of the reduction of DHA in that blood. There was also accordingly an increase in the rate of reduction.

b. Procedure.

Solutions of GSH and cysteine hydrochloride were prepared in which the concentration of sulphydryl groups was the same. (Note: 157.5mg. cysteine hydrochloride and 307mg. glutathione both contain 33.5mg. -SH.) Each was then added to a portion of a specimen of blood, previously saturated with CO, to give the /

the same final concentration of -SH groups in each portion. The reduction of DHA was followed in both, estimating the AA produced in each specimen after one hour's incubation at 37°C.

Results.

The results quoted in Table 16b showed that the hydrogen donor was the sulphydryl group and the reducing capacity was not a property peculiar to GSH.

c. Procedure.

A solution of egg albumin, prepared according to the instructions given by Kekwick and Cannon (1936) was reduced by electrolysis. A current of 60 milliamps. was passed through a 2% (w/v) solution of the albumin for 15 minutes. The albumin solution, which gave a positive reaction with sodium nitroprusside after reduction, showing that free -SH groups were present, was then added to one portion of a specimen of haemolysed, CO-saturated blood. The effect of this addition on the capacity of the blood to reduce DHA was then estimated. The incubation period was one hour at 37°C.

Results.

The result for one experiment is quoted in Table 16c. The addition of fixed protein -SH resulted in an increased DHA reduction.

d. Procedure. /

d. Procedure.

The choice of an -SH inhibitor was limited by its effect on the estimation for AA. Alloxan and the only arsenite salt available were both found to interfere with the indophenol method, but the addition of iodoacetate or of certain concentrations of cyanide had no effect on the estimation procedure.

Cyanide is a relatively unspecific reagent inhibiting several groups including -SH. Iodoacetate is regarded as a specific -SH inhibitor.

A portion of blood, after CO saturation, was divided into two parts. The appropriate inhibitor was added to one portion, and then DHA to both to give 10mg.DHA/100ml. mixture. The amount of AA produced after both portions had been incubated for one hour at 37°C was measured.

Results.

The figures listed in Table 16d showed that the addition of -SH inhibitors to the reduction medium destroyed the reducing capacity.

4. A Supplementary Reduction Mechanism.

Introduction.

Ascorbic acid in high concentrations was known to interfere with the iodiometric titration method of Woodward and Fry (1932) for glutathione. Estimations of glutathione by /

by this method in blood to which DHA had previously been added were therefore unreliable. Investigations were carried out on such blood by Bhattacharya (1955) in this laboratory using the glyoxalase method of estimation, employing a manometric technique. These analyses showed clearly that the reduction of DHA in blood did not result in any alteration in the concentration of blood GSH. There arose the concept of some other agent in the human erythrocyte which could also reduce added DHA, either independently of, or in conjunction with the endogenous GSH.

a. Procedure.

A haemolysed RBC suspension was prepared with erythrocytes and water. After CO saturation, portions of the suspension were diluted to different extents with phosphate buffer at pH 7.4. The concentration of GSH having been determined in the original suspension by means of the idiometric titration method, it was possible to supplement the diluted RBC-buffer mixtures with concentrated GSH solution in order that the final GSH concentration in each portion was the same. A very concentrated GSH solution was used in order to minimise the decay of GSH prior to addition to the blood mixtures. DHA was then added to all the portions to give a final concentration of 10 mg.DHA/100 ml. blood in each, and the amount of AA produced after thirty minutes' incubation at 37°C was estimated.

Results /

Table 17

The effect on dehydroascorbic acid reduction produced by the increase in RBC concentration, with a constant glutathione concentration

Estimated Results

		(a) Proportion RBC suspension (%)	(b) Initial GSH concentration (mg./100ml.)	(c) Final GSH concentration (mg./100ml.)	(d) AA produced (mg./100ml.)
1.	i	50	10.7	10.7	2.52
	ii	33	8.8	7.0	1.50
	iii	17	7.3	5.2	0.75
2.	i	50	6.1	4.6	1.32
	ii	33	5.5	4.3	1.05
	iii	17	6.1	3.3	1.00
3.	i	50	12.5	11.9	1.97
	ii	33	13.1	12.6	1.60
	iii	17	14.1	11.6	1.30
4.	i	50	11.4	9.2	2.03
	ii	33	11.0	8.2	1.51
	iii	17	10.9	8.7	1.13

Calculated Results

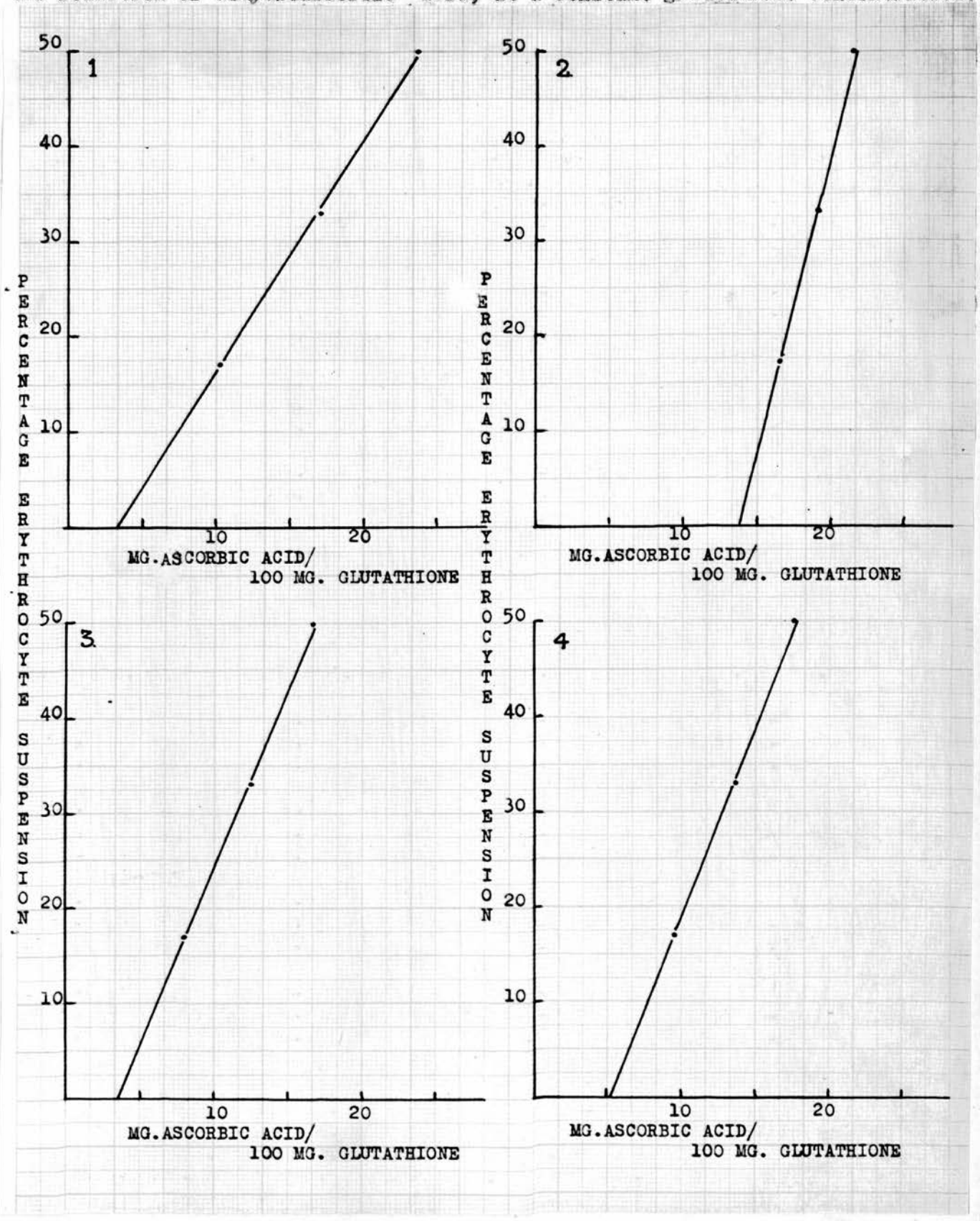
		(e) GSH loss (mg./100ml.)	(f) Theoretical GSH loss (mg./100ml.)	(g) AA/100mg. GSH (mg.)	(h) Difference (mg./100mg.)
1.	i	0	8.8	23.6	
	ii	1.8	5.2	17.0	6.6
	iii	2.1	2.6	10.3	6.7
2.	i	1.5	4.6	21.6	
	ii	1.2	3.7	19.1	2.5
	iii	2.8	3.5	16.4	2.7
3.	i	0.6	6.9	16.6	
	ii	0.5	5.6	12.5	4.1
	iii	2.5	4.6	8.0	4.5
4.	i	2.2	6.7	17.8	
	ii	2.8	4.9	13.7	4.1
	iii	2.2	3.5	9.6	4.1

Explanation of Calculations

Column (e) : Results in col.(b) - results in col.(c)
Column (f) : Results in col.(d) substituted in theoretical equation(see text)
Column(g) : (Result in col.(d)/Result in col.(b)) x 100
Column (h) : i Col.(g)i - col.(g)ii. ii Col.(g)ii - col. (g)iii

Figure 6

The relationship between the concentration of the erythrocyte suspension and the reduction of dehydroascorbic acid, at a constant glutathione concentration.



Results.

Results of several of these estimations are quoted in Table 17. By means of the calculations detailed in the table, a linear relationship was observed between the proportion of erythrocytes in the suspension and the AA produced for every 100 mg. GSH in the suspension. The relationship is shown graphically in Figure 6, for each experimental group. These graphs were all of the type $y = ax + b$. This indicated that, given a constant level of GSH, the reduction of DHA by an RBC-water suspension increased with an increase in the proportion of RBCs present.

Positive errors in the estimation of GSH were probable after incubation, due to the AA present in the specimen, and this would produce a negative error in the estimation of GSH loss. Nevertheless the error was not large enough in any of the examples to be the complete explanation of the difference between the actual values found and the theoretical values for GSH loss based on the equation,

$$DHA + 2GSH = AA + GSSG.$$

This led to the conclusion that, as the concentration of erythrocytes in a mixture increased, so less of the endogenous GSH was used in the reduction of DHA. This corroborated the manometric estimations for GSH performed on specimens of blood incubated with DHA.

b. Procedure /

Table 18

The effect on dehydroascorbic acid reduction produced by dialysis, and by glutathione supplementation of dialysates

Estimated Results

	Specimen	(a) Initial GSH concentration (mg./100ml.)	(b) Final GSH concentration (mg./100ml.)	(c) AA produced (mg./100ml.)	
	i Control	8.6	8.0	1.38	
1.	ii Dialysed + GSH	9.5	7.7	0.84	
	iii Dialysed	3.1	2.5	0.62	(45 hrs. dialysis
	i Control	9.8	9.2	1.23	
2.	ii Dialysed + GSH	7.7	4.4	2.10	
	iii Dialysed	1.2	1.2	0.94	(46 hrs.' dialysis
	i Control	12.9	8.6	2.46	
3.	ii Dialysed + GSH	16.3	12.6	2.06	
	iii Dialysed	5.8	4.6	1.45	(18 hrs.' dialysis
	i Control	12.6	8.6	2.58	
4.	ii Dialysed + GSH	16.0	11.2	2.78	
	iii Dialysed	4.9	4.0	1.58	(16 hrs.' dialysis
	i Control	8.6	8.6	1.53	
5.	ii Dialysed + GSH	11.3	5.8	1.17	
	iii Dialysed	3.1	2.1	0.57	(46 hrs.' dialysis

Calculated Results

	(d) AA due to added GSH (mg./100ml.)	(e) GSH loss (mg./100ml.)	(f) GSH loss in i and ii (mg./100ml.)	(g) Theoretical GSH loss in i and ii (mg./100ml.)
	i	0.6	0.6	4.8
1.	ii 0.22	1.8	1.2	0.8
	iii	0.6		
	i	0.6	0.6	4.3
2.	ii 1.16	3.3	3.3	4.0
	iii	0.0		
	i	4.3	4.3	8.6
3.	ii 0.61	3.7	2.5	2.1
	iii	1.2		
	i	4.0	4.0	9.0
4.	ii 1.20	4.8	3.9	4.2
	iii	0.9		
	i	0.0	0.0	5.3
5.	ii 0.60	5.5	4.5	2.1
	iii	1.0		

Explanation of Calculations

Column (d) : Col.(c)ii - col.(c)iii
Column (e) : Results in col.(a) - results in col.(b)
Column (f) : i)--col.(e)i ii)--col.(e)ii - col.(e)iii
Column (g)i: Results in col.(c)i substituted in theoretical equation,
$$DHA + 2GSH = AA + GSSG$$
Column (g)ii: Results in col.(d)ii substituted in theoretical equation

b. Procedure.

A haemolysed suspension of erythrocytes was prepared with water and a portion of this was dialysed. Cold distilled water was used to minimise the interference of cupric ions. The duration of the dialysis ranged from 16 to 46 hours in the different experiments. The rate of flow of the water in the dialysis was 10 litres/24 hours. Iodiometric titrations were carried out to determine the extent to which GSH had disappeared from the dialysed suspension. The remainder of the haemolysed suspension was kept at 4° C to be used as a control. The volume of the control was adjusted with distilled water until the haemoglobin concentration in both it and the dialysate were the same. The haemoglobin was estimated using the alkaline haematin method of Clegg and King (1942).

After saturation of both portions with CO, concentrated GSH solution was added to a portion of the dialysed specimen to increase the GSH concentration to a value approximating to that in the control. DHA was then added to the specimens to give a final concentration of 10 mg./100 ml. in each and the AA formed after 30 minutes' incubation at 37° C was estimated. Iodiometric titrations for GSH were carried out on each of the 3 specimens before and after incubation.

Results.

Several experiments were carried out and the findings are reported in Table 18. Dialysis lowered the GSH concentration /

concentration very considerably, although complete removal was not achieved on any occasion. There was the possibility of a negative error in the estimation of GSH loss (cf. 4a Results above). Nevertheless, the discrepancy between the observed and theoretical values for GSH loss in the undialysed samples was too great to be explained as experimental error. The actual loss of GSH in the solution was very much less than was expected on the basis of the theoretical relationship between DHA and GSH.

The same was not true for the dialysed samples which had been supplemented with added GSH. There was only a small difference between the value found and the calculated value for GSH loss and this was within experimental error. Therefore, in dialysed mixtures which had been supplemented with GSH, the loss of GSH during the reduction of DHA was that expected on the basis of the theoretical relationship between the two substances.

c. Procedure.

A haemolysed suspension of erythrocytes, prepared with water, was dialysed against running distilled water for 48 hours. After saturation with CO, part of the dialysate was reduced electrolytically with a current of 60 milliamps. for 10 minutes. A longer period of reduction was not possible since the KCl bridge became completely blocked with RBC protein. It was assumed on analogy with plasma that the reduction /

Table 19

The effect on dehydroascorbic acid reduction produced by electrolytic reduction in dialysed and glutathione-supplemented RBC-water suspensions

Estimated Results

	Specimen	(a) Initial GSH concentration (mg./100ml.)	(b) Final GSH concentration (mg./100ml.)	(c) AA produced (mg./100ml.)
	i Dialysed contl.	8.1	5.5	0.20
1.	ii Dialysed + GSH	20.4	14.4	1.06
	iii Dialysed, reduced + GSH	18.3	12.3	1.62
	i Dialysed contl.	1.7	1.2	0.21
2.	ii Dialysed + GSH	16.0	14.1	1.05
	iii Dialysed, reduced + GSH	17.0	14.7	1.43

Calculated Results

	(d) GSH loss (mg./100ml.)	(e) Theoretical GSH loss (mg./100ml.)	(f) GSH loss in ii and iii (mg./100ml.)	(g) Theoretical GSH loss in ii and iii (mg./100ml.)
	i 2.6	0.7		
1.	ii 6.0	3.7	3.4	3.0
	iii 6.0	5.7	3.4	5.0
	i 0.5	0.7		
2.	ii 1.9	3.7	1.4	3.0
	iii 2.3	5.0	1.8	4.3

Explanation of Calculations

Column (d) : Result in col.(a) - result in col. (b)
Column (e) : Result in col.(c) substituted in theoretical equation,
DHA + 2GSH = AA + GSSG
Column (f) : ii Result in col.(d)ii - result in col.(d)i
iii Result in col.(d)iii - result in col.(d)i
Column (g) : ii Result in col.(e)ii - result in col. (e)i
iii Result in col.(e)iii - result in col.(e)i

reduction liberated some, if not all, of the protein -SH groups. Qualitative estimation with nitroprusside was out of the question due to the colour of the suspension. Quantitative estimation for liberated -SH groups was not possible since all the methods for -SH groups so far investigated involved protein precipitation as a preliminary.

The reduced portion and another portion of the dialysate were then supplemented with concentrated GSH to give the same final GSH concentration in both. A third portion was used as a control. Production of AA from DHA added to the three specimens was estimated, also the fall in GSH concentration during the period of the reduction. The initial concentration of DHA was 10 mg./100 ml. in each specimen and the period of incubation at 37°C lasted 30 minutes.

Results.

The results for two experiments are given in Table 19. The GSH loss from the specimens supplemented with the tripeptide was the same in both cases. The production of AA in the specimen which had been previously reduced electrolytically was greater than that in the unreduced specimen. The loss of GSH corresponding to the AA production, using the theoretical equation as a basis of calculation, was therefore greater in the electrolytically reduced specimen than in the other. The loss of GSH, expected on theoretical grounds, exceeded the loss actually found in the reduced specimen to

an /

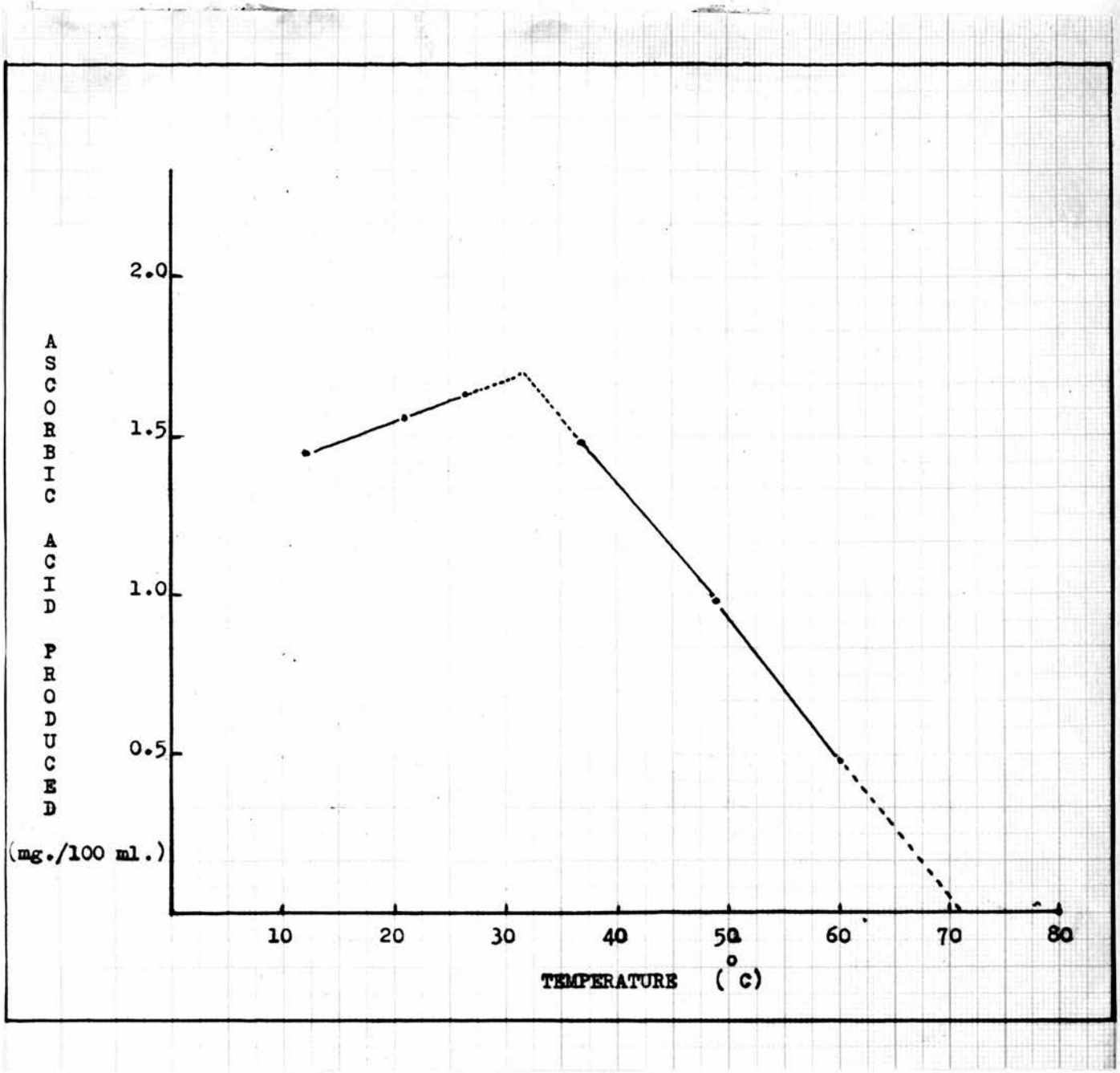
Table 20

The effect of temperature on dehydroascorbic acid reduction

Temperature (°C)	AA produced (mg./100ml.)
12°	1.41
21°	1.62
26.5°	1.76
37°	1.48
49°	0.96
60°	0.46
80°	0.0

Figure 7

The effect of temperature on
the reduction of dehydroascorbic acid.



an extent beyond the limits of experimental error, and suggested that the liberation of free -SH groups on the protein resulted in a lessened demand for GSH in DHA reduction.

5. Factors Influencing the Reduction Mechanism.

(a) Temperature:

Procedure.

Portions of the same haemolysed CO-saturated 30% RBC suspension were incubated for 30 minutes with a concentration of 10 mg./100 ml.DHA at several different temperatures. The extent of the reduction was determined in each.

Results.

From the results expressed in Table 20 and Figure 7, the Q_{10} (15° - 25°) of the reduction of DHA was found to be 1.12 below 30° C. Above 30° C, the temperature at which the rate of the reduction was maximal, the Q_{10} (35° - 45°) was calculated to be -1.43.

(b) Hydrogen Ion Concentration.

Procedure.

Portions of the same haemolysed CO-saturated RBC suspension were incubated at different hydrogen ion concentrations. The pH of the portions was adjusted using NHCl, N Na.$\overline{OAc}$, 0.2M KH_2PO_4, or 0.2N NaOH solutions. The reduction of DHA at an initial concentration of 10 mg./100 ml. was estimated in each specimen after 90 minutes' incubation at 37°C.

Results /

Figure 8

The effect of hydrogen ion concentration on reduction of dehydroascorbic acid.

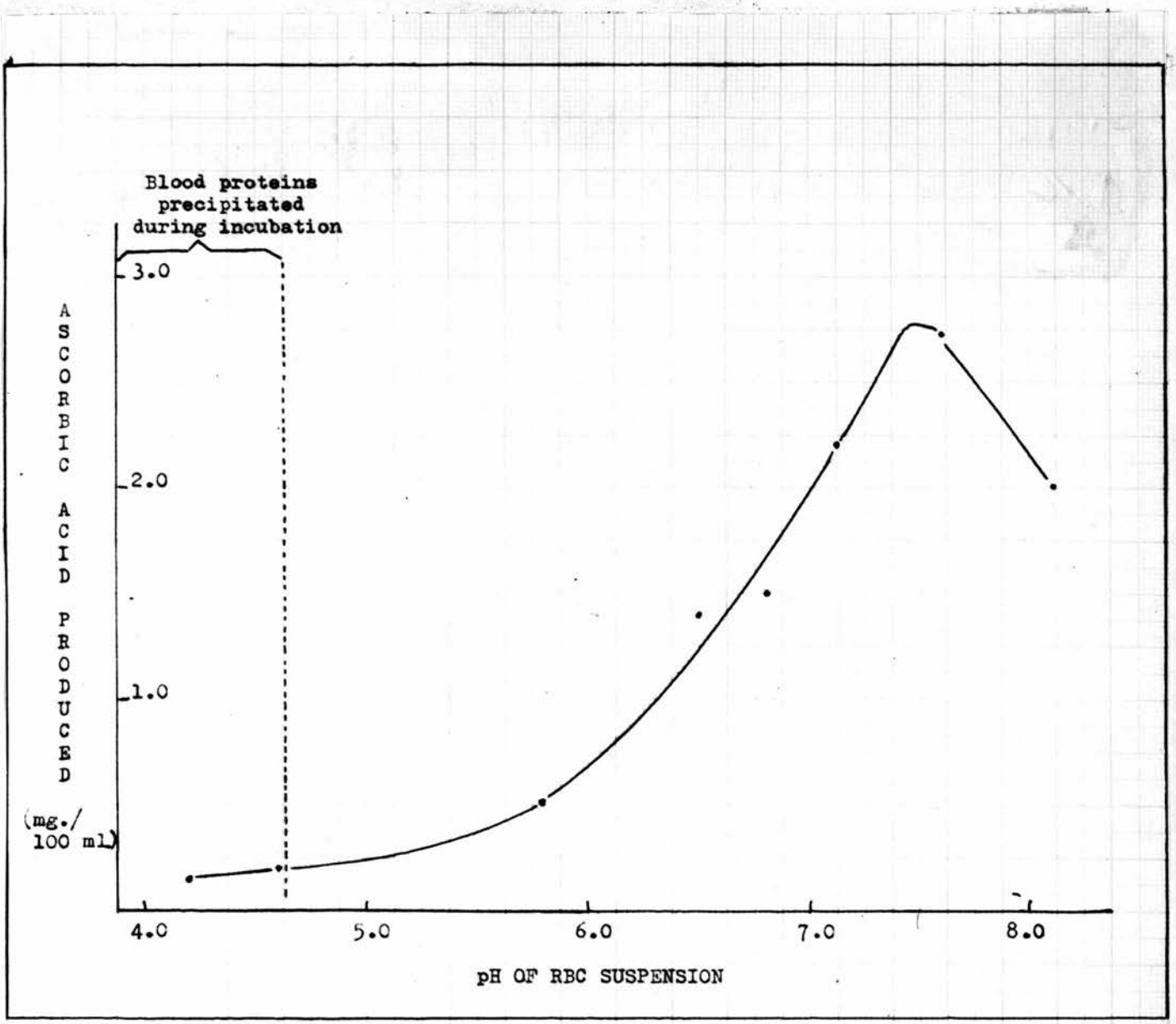


Table 21

The effect of an increase in dehydroascorbic acid concentration on the dehydroascorbic acid reduction in blood

DHA concentration (mg./100ml.)	Aa produced (mg./100ml.)
5	1.80
10	2.70
25	4.26
50	5.00
100	5.40
150	5.80
200	6.00

Figure 9

The effect of dehydroascorbic acid concentration on the extent of the reduction.

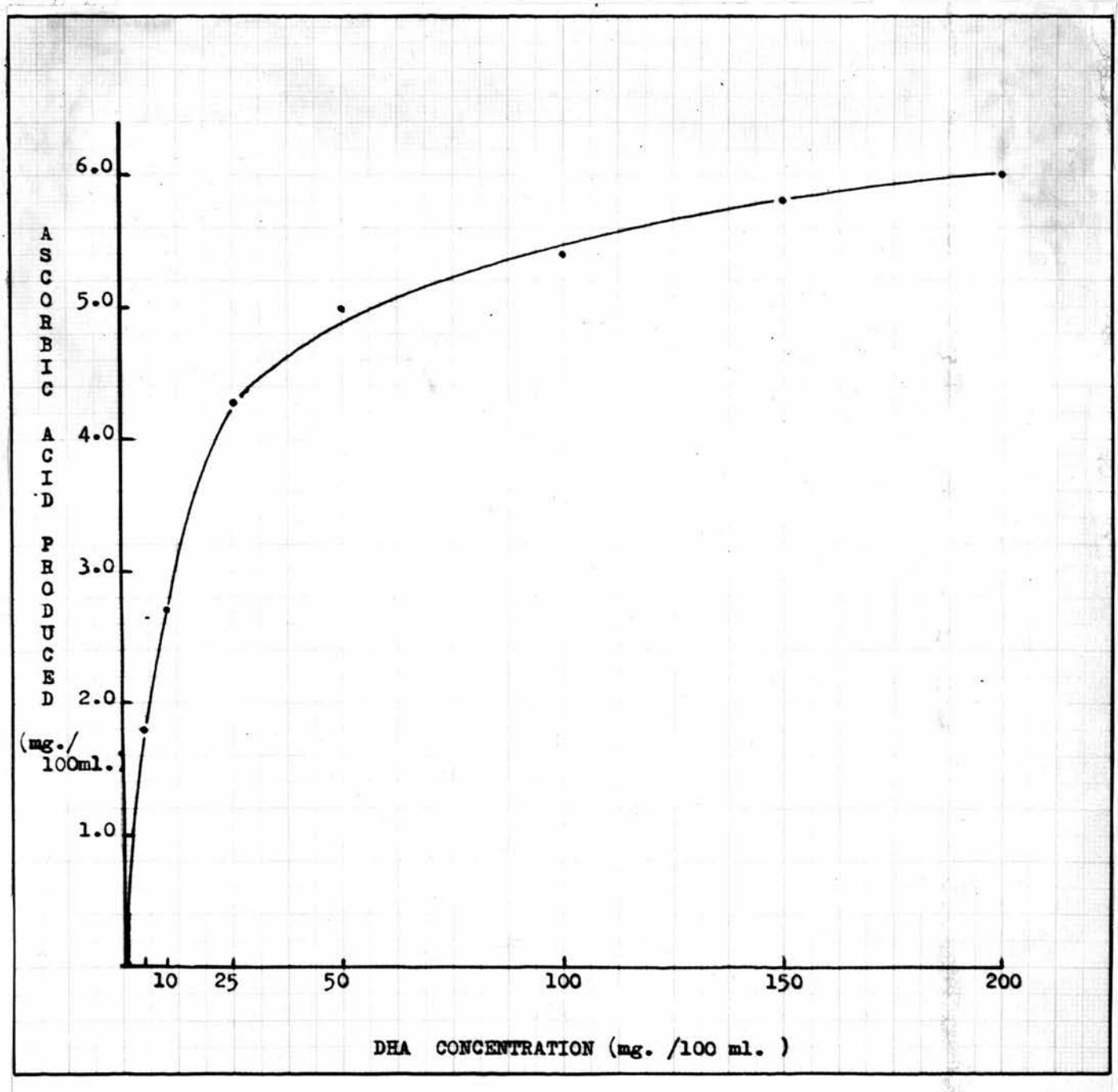
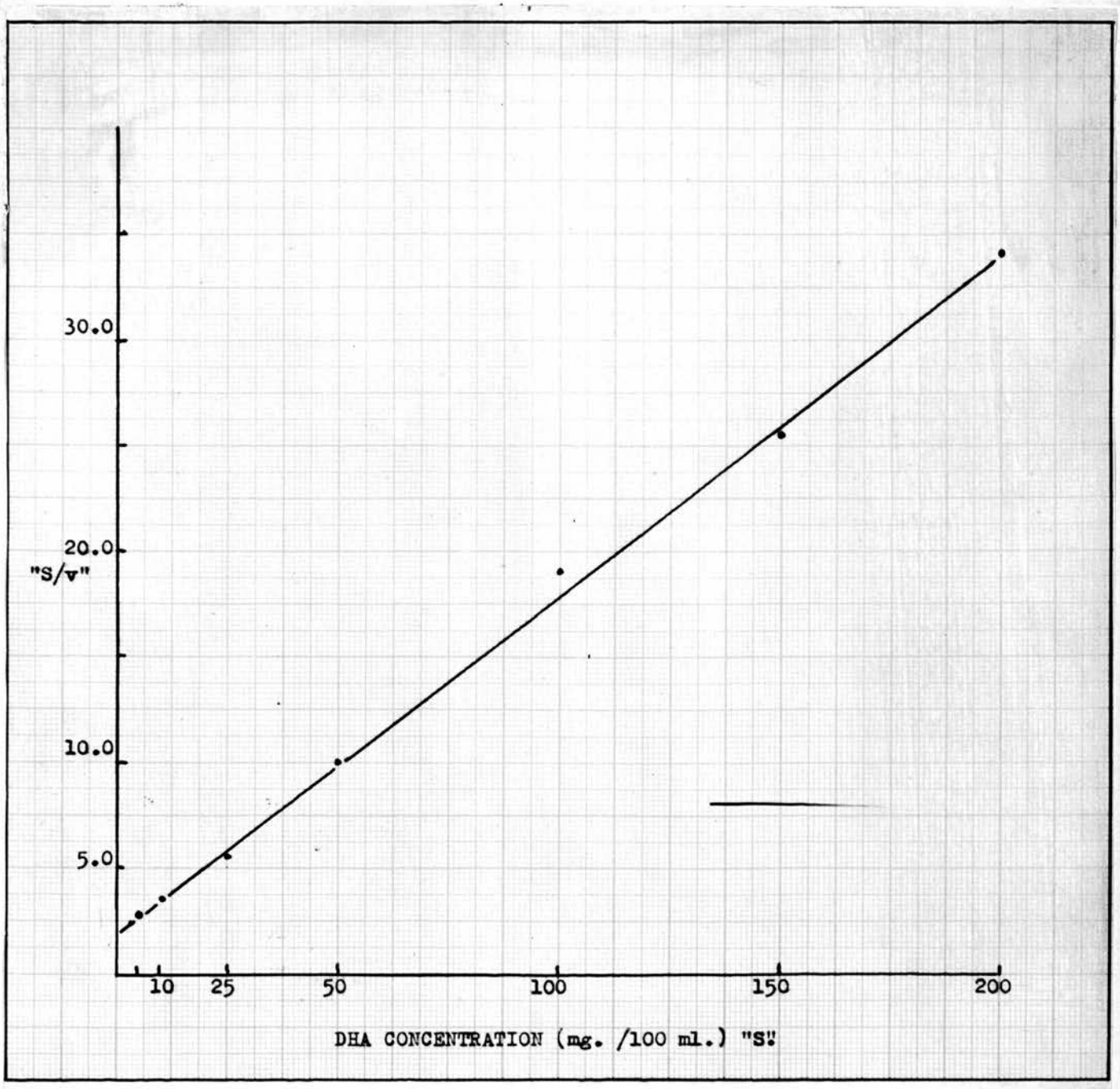


Figure 10

Graphical representation of Lineweaver and Burk's (1934) equation for the reduction of dehydroascorbic acid in relation to its concentration.



Results.

Figure 8 shows the effect of the change in pH on the rate of the reduction. The rate was maximal within the physiological pH range, pH 7.4-7.6.

(c) DHA Concentration:

Procedure.

Portions of an erythrocyte suspension in water, with a PCV approximating to 30%, were completely haemolysed and saturated with CO. Each portion was incubated for 30 minutes at 37°C at a different concentration of DHA, and the extent of the reduction to AA was estimated.

Results.

The results are tabulated in Table 21 and plotted in Figure 9. The Michaelis constant, K_m, for the reaction at this erythrocyte concentration was calculated using Lineweaver and Burk's (1934) equation and was found from Figure 10 to be 12.5 mg./100 ml. blood (0.0007 M). The calculation of the equation and the derivation of K_m are shown in Appendix II.

Discussion.

Investigation of the reduction mechanism in blood with regard to DHA resulted in the formulation of the following conclusions:

1. The reducing mechanism is located within the erythrocyte /

erythrocyte.

2. The extent of reduction is characteristic of the blood under examination and is independent of the initial DHA concentration.

3. The hydrogen donor for the reduction is a sulphydryl group, either in the form of a soluble -SH donor or as "fixed" SH.

4. The extent of the reduction is directly proportional to the erythrocyte concentration when the GSH concentration is maintained at a constant value.

5. Dialysis of blood either destroys or removes some factor concerned in the reduction of DHA.

6. Electrolytic reduction of dialysed blood partially restores the reducing capacity lost on dialysis.

7. There is an optimal temperature of 30°C.

8. Hydrogen ion concentration influences the reduction, the rate being maximal in the physiological pH range.

9. The rate of the reduction is proportional to the initial DHA concentration, below a specific concentration. Above this DHA concentration, the rate is independent of the concentration.

It has therefore been confirmed that the reduction of DHA is controlled by -SH groups; it has also been confirmed that the reduction does not follow the theoretical relationship

$$DHA + 2GSH = AA + GSSG.$$

The /

The contributory or supplementary reducing mechanism has been found to be associated with the erythrocyte. The only source of -SH groups in the RBC other than GSH is the protein, either globin of haemoglobin or other protein present in the RBC. The sensitivity of the system to temperature, to pH, and to dialysis, suggests that protein is concerned in the reduction. The loss of activity upon dialysis is not due merely to the removal of GSH from the blood suspension since addition of GSH to the dialysate does not restore the original situation. Dialysis removes other factors of low molecular weight which may be concerned in the reduction, such as any diphospho- or triphosphopyridine-nucleotide present in the medium. Prolonged dialysis probably also causes a certain amount of protein denaturation, including the oxidation of free -SH groups.

It therefore seems that the reduction of DHA is brought about by endogenous GSH, the concentration of which is kept constant by regeneration from an additional reduction mechanism, probably the fixed -SH groups of the RBC protein. The question arises whether the regeneration of GSH from GSSG is governed enzymically.

Roe and Barnum (1936) suggested that tissue reduction of DHA was enzymically controlled, but the evidence they advanced to support this hypothesis was subsequently discounted by Borsook et al. (1937). Schultze et al. (1937) reported the existence /

existence of a heat-stable reducing mechanism for DHA in liver tissue. Kinkawa's (1944) report of a heat-stable non-enzymic DHA reduction mechanism in tissues has not been confirmed in this study on blood. The evidence from the present investigation has shown that heating at 100°C completely destroys the reducing capacity.

The evidence collected in this investigation suggests, on balance, the participation of an enzyme or an enzyme system. Protein is involved in the reaction and so the mechanism exhibits many enzyme characteristics, such as response to temperature, pH, and heavy metals. The K_m is small in value and the reaction is rapid.

Indirect support for the possible existence of an enzyme system concerned with DHA reduction is found in the report by Rall and Lehninger (1952) of an enzyme in rat liver, glutathione reductase, which catalyses GSSG reduction, with $TPNH_2$ as co-enzyme.

$$TPNH_2 + GSSG = TPN + 2GSH$$

This enzyme may be part of an enzyme chain responsible for the reduction of DHA in blood. It has not however been demonstrated to occur in human blood: also there is reported to be only a very limited concentration of TPN in human blood.

GENERAL DISCUSSION

GENERAL DISCUSSION

The evidence collected in this report from work on human blood agrees with the reports in the literature that mammalian tissues, among them red blood cells, have a reducing capacity with regard to added dehydroascorbic acid.

ERYTHROCYTE REDUCTION OF DEHYDROASCORBIC ACID.

The reduction of DHA in the erythrocytes is in the first instance brought about by glutathione within the RBCs. The GSH concentration of these cells is therefore a contributory factor controlling the extent of the reduction. GSSG, produced in the course of this reaction, is reduced again and so becomes available to reduce more DHA. It is still not clear what mechanism is responsible for the regeneration of GSH from GSSG.

1) GSSG may be reduced by means of an interaction with one or other of the phosphopyridinenucleotides. Such a reduction of GSSG by $TPNH_2$ is reported to occur in rat liver tissue, catalysed by the enzyme glutathione reductase (Rall and Lehninger, 1952). This enzyme has not been found in erythrocytes, but the possibility of its presence, or of a parallel system, must be borne in mind.

2) Alternatively, GSSG may be reduced to GSH by direct interaction with the fixed -SH groups on the erythrocyte protein. /

protein. Since the reaction is sensitive to -SH activators and inhibitors and also to protein denaturating agents, it seems probable that the erythrocyte protein is involved in the reaction. The mechanism of regeneration of such fixed -SH groups, when they have been used in the reduction of GSSG, is quite unknown.

3) A third possible means of regeneration of GSH might be through the agency of a phosphopyridinemucleotide-enzyme system (such as glutathione reductase-TPNH) which, in turn, would accept hydrogen from protein -SH groups. Alternatively, the steps in the reaction chain might be reversed, GSSG being reduced by protein -SH groups, the latter being restored to their original state by means of a "reductase" enzyme, in conjunction with one or other of the phosphopyridinemucleotides.

SIGNIFICANCE OF ERYTHROCYTE REDUCTION OF DEHYDROASCORBIC ACID.

Although the reduction capacity of erythrocytes for DHA has been demonstrated under *in vitro* conditions in an isolated biological system, it is another matter whether it is justifiable to assume that the same response occurs and is biologically important *in vivo*.

In the experiments described earlier in this thesis, the reduction of DHA by erythrocytes was examined and the conclusions regarding the mechanism were reached with non-physiological /

non-physiological experimental conditions. An exceedingly high concentration of DHA was used, far above that which could occur naturally in blood; the blood was saturated with CO; most of the leucocytes were removed prior to the investigation.

The results in Table 21 and Figure 9 showed that the initial rate of reduction of DHA was a function of the concentration of the added DHA. The extent of the reduction was independent of the initial DHA concentration when the latter was greater than 50 mg./100 ml. blood. Table 15 showed that in all cases, with DHA concentrations greater than 10 mg./100 ml. blood, the reduction was incomplete and ceased even in the presence of considerable amounts of GSH. This was not due to exhaustion of the reducing mechanism, since the addition of more DHA to the medium resulted in the production of more AA. Figure 9 showed, however, that at very low concentrations of DHA, such as might be found in blood, the major portion of the added substance was reduced to AA.

The use of a CO atmosphere was introduced for technical reasons and had no influence on the behaviour of DHA after addition to whole blood. The CO atmosphere prevented a coupled oxidation of AA and haemoglobin when the cells were lysed, (Lemberg et al., 1939), and so enabled the quantitative estimation of AA to be made in whole blood. The results in Tables /

Tables 8a and 8b showed that DHA passed into the erythrocytes equally as well in aerobic conditions as in blood previously saturated with CO. Plasma analyses, quoted in these tables, showed that AA appeared in the plasma after DHA addition to whole blood in an oxygen or in a CO atmosphere. Reduction of DHA was therefore realised to occur in blood, regardless of the atmosphere in which the blood was maintained and by analysis of the CO-saturated erythrocytes, it was shown that the erythrocytes were the active elements in the reduction.

Leucocytes contain a high concentration of Total AA (Lowry et al., 1946). They are also reported to oxidise AA to DHA (Lloyd, 1951), and their presence in blood influences the transfer of ascorbic acid across the blood cell membrane (Heinemann, 1941). In light of their metabolic activity towards ascorbic acid, it was therefore necessary to remove them from all blood samples examined, in order that the behaviour of the erythrocytes with regard to AA and DHA could be examined accurately.

Taking the evidence as a whole, there is every reason to believe that the conclusions reached, using the non-physiological experimental conditions described, are valid also for the conditions found in vivo.

The experiments in which DHA was fed, of which details were given in Tables 5 and 6, showed that administered DHA was rapidly reduced within the body and so corroborated the results obtained /

obtained by *in vitro* investigations. The reduction occurs within the red blood cells, but this does not preclude the existence of sites of reduction in many other organs and tissues. This is confirmed by the evidence of several workers, including Todhunter *et al.* (1950) and Clayton *et al.* (1954), from investigations carried out on human subjects.

EXISTENCE OF DHA IN PLASMA.

The experiments in which plasma was treated with *Esch. coli* suspension demonstrated that DHA is found in human blood plasma. It is not invariably possible to detect or measure it in specimens of plasma, but the great majority of samples examined contained concentrations of DHA ranging from 0.05 - 0.25 mg./100 ml. plasma. It did not at first seem possible to reconcile the existence of DHA in the plasma with the rapid reducing capacity of erythrocytes and other tissues for that substance. These two seemingly contradictory conditions can however be recognised to be complementary, by means of the following hypothesis. In the construction of this, the metabolism of the leucocytes with respect to AA and DHA is taken into consideration. The leucocyte response to vitamin C was described by Heinemann (1941) and by Lloyd and Sinclair (1953).

POSTULATED /

POSTULATED METABOLISM OF ASCORBIC ACID IN HUMAN BLOOD.

Ascorbic acid passes into the blood from, for instance, the small intestine during absorption after ingestion. It is partly taken up by the leucocytes and there rapidly converted to DHA, with the loss of two hydrogen atoms. This may possibly, although not necessarily, occur through the intermediate of the free radical, monodehydroascorbic acid (Weissberger and LuValle, 1944). DHA then passes out of the leucocytes into the plasma whence several routes for its utilisation and further metabolism are available.

1) It may pass into the erythrocytes and there undergo reduction, the extent of which is characteristic of the blood under consideration. Estimations of the reduction capacity have been carried out on specimens of blood obtained from normal healthy subjects, considerable periods of time elapsing between two estimations on the same patient. It was found that the extent of the reduction of DHA was relatively constant for each individual, even if several months passed between one determination and the next.

After DHA has been reduced in the erythrocytes, the AA produced passes out of the RBCs to a small extent. Part of the AA remaining in the RBCs may be re-oxidised, possibly through the monodehydroascorbic acid radical to DHA, and so re-enter the GSH reduction mechanism.

2) Plasma DHA may be oxidised to CO_2 and water. This may /

may occur within the blood cells directly, particularly in the leucocytes, or in some other body tissue.

3) DHA may remain in the plasma to be converted to DGA and then finally to water and CO_2. Estimations suggest that this is, at most, only a minor route of DHA metabolism.

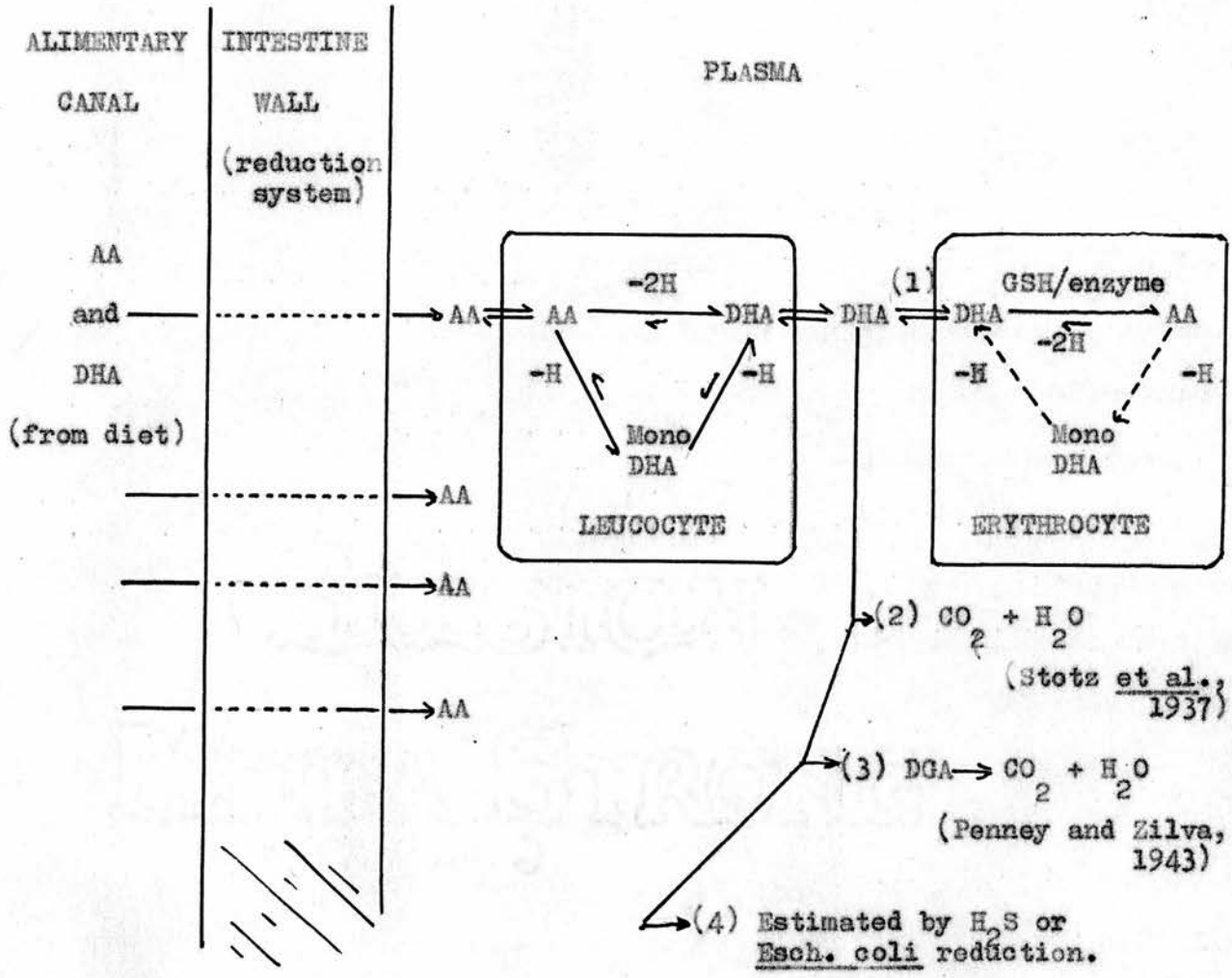
In normal conditions, the first pathway described for the metabolism of the DHA passing out of the leucocytes seems to be the only one of importance in its utilisation. In such a mechanism there is a small concentration of DHA likely to be present in the plasma, which will therefore be detectable by H_2S or Esch. coli reduction.

The second route is probably the chief means of catabolising DHA in the body. A mechanism for oxidising AA to water and CO_2 has been reported to be present in guinea-pig liver by Stotz et al. (1937). A similar mechanism probably occurs in human liver and other tissues.

The third pathway for DHA disposal is not thought to be important normally. There is no evidence that DGA is a component of human blood plasma in vivo. Penney and Zilva (1943) presented results that indicated the appearance of a small amount of DGA in guinea-pig blood plasma after feeding abnormally large doses of DHA. In the conditions found in the experiments on isolated blood specimens, where there is a high concentration of added DHA incubated in a closed metabolic system, the residual DHA is probably converted in large part to /

Figure 10

Diagrammatic representation of the behaviour of ascorbic acid in blood, in vivo.



to DGA as suggested by Lloyd and Parry (1954). A very different situation prevails *in vivo* where other routes for metabolising DHA, and many other complicating factors occur.

Figure 11 is a diagrammatic representation of the metabolism of AA and DHA postulated above. This hypothesis is recognised to be a crude explanation of the experimental evidence. At present, no satisfactory account can be developed for the abnormal behaviour with regard to DHA in blood from patients with various pathological conditions.

It is realised that other routes may exist for the utilisation and disposal of ascorbic acid in human blood in addition to that outlined above. Plasma AA may be directly oxidised to water and CO_2, without passing through the intermediate step of DHA; alternatively, plasma AA may lose one hydrogen atom to yield monodehydroascorbic acid, and in this form enter many metabolic reactions.

THE REGULATION OF DEHYDROASCORBIC ACID CONCENTRATION IN HUMAN BLOOD.

The factors in human blood which are therefore regarded as responsible for regulating the DHA content of human blood are;

1) the reduction capacity of the blood with regard to DHA. This is dependent on

a) /

a) the GSH concentration

b) the mechanism for GSSG reduction in the RBC

2) the leucocyte concentration, and

3) the ascorbic acid load in the diet.

SUMMARY

SUMMARY

1. Evidence has been presented to confirm previous reports in the literature that dehydroascorbic acid is a normal constituent of human blood plasma. The estimation procedure was a modification of one originally devised for use in plant analyses, and had not previously been applied to animal tissues.

2. Oral administration of dehydroascorbic acid solutions to human subjects resulted in an increased ascorbic acid concentration in the plasma, and so confirmed the previous reports of a biological reducing mechanism for dehydroascorbic acid. The results obtained by incubating fresh isolated suspensions of erythrocytes in plasma or saline with high concentrations of dehydroascorbic acid provided additional evidence of the existence of this mechanism in erythrocytes.

3. The extent of the reduction of dehydroascorbic acid was examined in blood from normal subjects and also from patients suffering from a variety of pathological conditions. The percentage of dehydroascorbic acid reduced to ascorbic acid under standard experimental conditions was estimated in each sample of erythrocyte-plasma suspension. The results obtained thus could be compared. In normal subjects, the values for the extent of the reduction were found to fall within a narrow range, (46% - 55% of the added DHA). The range of results was much wider in the various groups of pathological conditions examined. /

examined. There was a tendency for the extent of the reduction to be inversely related to the haemoglobin concentration of the original blood sample. This relationship was most marked in the specimens from patients with untreated Addisonian pernicious anaemia. Treatment of these patients with vitamin B_{12} did not result in a marked alteration in the behaviour of the blood with regard to dehydroascorbic acid.

The extent of the reduction of dehydroascorbic acid in blood drawn from patients with diabetes mellitus could not be related to the severity of the diabetic condition nor to the state of insulin control of the patient.

4. A study has been made of the mechanism responsible for the reduction of dehydroascorbic acid in human erythrocytes. The reduction is brought about in the first instance by endogenous glutathione. The glutathione is maintained in the reduced state, possibly through the agency of an enzyme system and with the erythrocyte -SH protein groups as hydrogen donors. The ultimate hydrogen donor is unknown. Glutathione reductase and one or both of the phosphopyridinenucleotides are possible components of such an enzyme system.

5. This reduction mechanism has been integrated into a hypothetical representation of the metabolism of ascorbic and dehydroascorbic acids in the leucocytes and erythrocytes in human blood. The possibility that monodehydroascorbic acid may be involved has been noted.

ACKNOWLEDGEMENTS

The work reported in this thesis was carried out in the Clinical Laboratory, Royal Infirmary, Edinburgh, under the guidance of Dr. C. P. Stewart, in collaboration with other research workers in the department. I am deeply indepted to Dr. Stewart and to Dr. J. S. Robson for their continual advice and encouragement.

I am also grateful to the staff of Wards 21 and 27 and the Bacteriology Department in the Royal Infirmary for their assistance and co-operation.

My thanks are due to the Department of Health for Scotland for the full-time grant by means of which this research was undertaken.

APPENDICES

APPENDIX I

THE METHOD FOR THE CALCULATION OF THE PERCENTAGE REDUCTION OF DEHYDROASCORBIC ACID.

Symbols.

Ascorbic acid, in plasma $\equiv (AA)_{plasma}$

Ascorbic acid, in cells $\equiv (AA)_{cells}$

Total Ascorbic acid, in plasma $\equiv (Total)_{plasma}$

Total Ascorbic acid, in cells $\equiv (Total)_{cells}$

$(AA)_{plasma}$, expressed in mg./100 ml. blood $\equiv$

$$\frac{\text{Estimated concentration AA} \times (100 - \text{PCV})}{100}$$

$(AA)_{cells}$, expressed in mg./100 ml. blood $\equiv$

$$\frac{\text{Estimated concentration AA} \times \text{PCV}}{100}$$

$(Total)_{plasma}$, expressed in mg./100 ml. blood $\equiv$

$$\frac{\text{Estimated concentration Total AA} \times (100 - \text{PCV})}{100}$$

$(Total)_{cells}$, expressed in mg./100 ml. blood $\equiv$

$$\frac{\text{Estimated concentration Total AA} \times \text{PCV}}{100}$$

$(AA)_{blood} = (AA)_{plasma} + (AA)_{cells}$

$(Total)_{blood} = (Total)_{plasma} + (Total)_{cells}$

$$\text{Percentage Reduction of DHA} = \frac{(AA)_{blood}}{(Total)_{blood}} \times 100$$

A worked example, employing the calculation, follows:

Sample /

Sample of blood (PCV = 30%), containing a final concentration of 10 mg./100 ml. blood DHA.

Readings.

AA, in plasma = 0.70 mg./100 ml. plasma

AA, in cells = 15.0 mg./100 ml. cells

Total AA, in plasma = 5.71 mg./100 ml. plasma

Total AA, in cells = 20.0 mg./100 ml. cells

Calculation.

$(AA)_{plasma} = \frac{0.70 \times (100 - 30)}{100}$ mg./100 ml. blood

= 0.49 mg./100 ml. blood.

$(AA)_{cells} = \frac{15.0 \times 30}{100}$ mg./100 ml. blood

= 4.50 mg./100 ml. blood.

$(AA)_{blood}$ = (0.49 + 4.50) mg./100 ml. blood

= 4.99 mg./100 ml. blood.

$(Total)_{plasma} = \frac{5.71 \times 70}{100}$ mg./100 ml. blood

= 3.99 mg./100 ml. blood.

$(Total)_{cells} = \frac{20.0 \times 30}{100}$ mg./100 ml. blood

= 6.00 mg./100 ml. blood.

$(Total)_{blood}$ = (3.99 + 6.00) mg./100 ml. blood

= 9.99 mg./100 ml. blood.

percentage reduction of DHA $= \frac{4.99}{9.99} \times 100\% = 49.9\%$

APPENDIX II

THE DERIVATION OF LINEWEAVER AND BURK'S (1934) EQUATION.

Michaelis equation : $v = \frac{Vs}{S + K_m}$

$v \equiv$ AA produced/30 min.

$S \equiv$ DHA concentration.

$K_m \equiv$ Michaelis constant.

$V \equiv$ limiting concentration of AA produced/30 min.

From this, Lineweaver and Burk's equation can be derived.

Taking reciprocals : $\frac{1}{v} = \frac{K_m}{V} \times \frac{1}{S} + \frac{1}{V}$

Multiply by "S" : $\frac{S}{v} = \frac{K_m}{V} + \frac{S}{V}$

Application to DHA Reduction.

DHA "S" mg./100 ml.	AA "v" mg./100 ml.	$\frac{S}{v}$
5	1.8	2.8
10	2.7	3.7
25	4.3	5.75
50	5.0	10.0
100	5.4	19.0
150	5.8	25.5
200	6.0	34.0

Lineweaver /

Lineweaver and Burk's equation : $\frac{S}{v} = \frac{K_m}{V^m} + \frac{S}{V}$

From above table and graph in Figure 1D,

$$10 = \frac{K_m}{V} + \frac{50}{V} \qquad (1)$$

$$20 = \frac{K_m}{V} + \frac{112.5}{V} \qquad (2)$$

$$10V = K_m + 50 \qquad (1)$$

$$20V = K_m + 112.5 \qquad (2)$$

Solution of these equations gives :

$K_m = 12.5$ $\qquad$ $V = 6.25$ mg./30 min.

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